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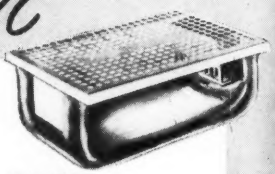
Published quarterly by the
FEDERATION OF AMERICAN SOCIETIES
for EXPERIMENTAL BIOLOGY

Leading Laboratories Prefer MOUSE-HOUSE



THE *Plastic* ANIMAL CAGE

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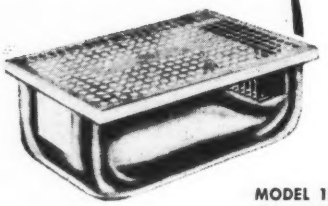


1. **SPEEDS RESEARCH**—clear, plastic walls permit daily cage inspection, aid observation of rodent development. False mesh bottoms speed metabolism tests and keep animals clean.

2. **CLEANER** — lower portion of cage is free from crevices and corners, thus cleaning usually involves only (1) turning cage upside down and (2) brushing unclean surfaces with hot soap suds and rinsing in hot water. Body acids and urine do not affect plastic cages. We recommend our HYTRON as a disinfectant.

3. **SAFER** — slow changing temperatures of smooth plastic walls protect rodents against drafts, and sudden temperature changes, thus reducing mortality rate.

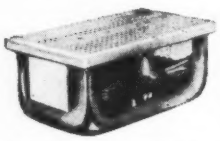
4. **VERMIN PROOF** — the plastic MOUSE-HOUSE automatically eliminates the outside enemies to highly technical research.

5. **LESS COSTLY** — by comparison with utility value of other cages. With reasonable handling these cages last indefinitely, making them more economical.

MODEL 150
STANDARD

CLEAR PLASTIC CAGE FOR RATS AND HAMSTERS. Molded from crystal clear plastic sheets; interchangeable stainless steel perforated cover fits snugly on the fluted sides of cage and has two special openings for inserting drinking tubes. Stainless steel feed cup fits over the end of the cage, inside the top rim, has a slotted base and a suspended hood. Permanent record card on end. Cage size for all 150 Models 11½" x 18½" x 6½".

Follow the lead of a large majority of research departments in leading universities, hospitals, privately endowed institutions and government bureaus who are regular users of this modern plastic animal cage. Everyone who uses this practical economical way of caging experimental rodents say the MOUSE-HOUSE cage is superior in every way.

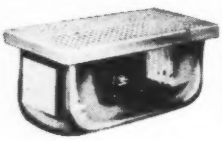


MODEL 101

CLEAR PLASTIC STOCK CAGE FOR MICE. Has all the construction features described for Model 150, but is smaller. Accommodates small rodents, cage size 11½" x 7½" x 5".

CLEAR PLASTIC CAGE FOR RATS AND HAMSTERS. Has same features of other plastic cages plus a stainless perforated slide-on cover with built-in food hopper which can be filled without opening the cage, plus a removable expanded metal grate above the plastic bottom. Grate is removable for cleaning. Size 13" x 9½" x 7".

MODEL 110

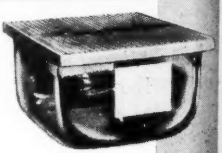


MODEL 103 STANDARD

CLEAR PLASTIC CAGE FOR MICE. This cage has all the construction features described for Model 101 except the size is smaller. Cage size for all 103 Models 11⅜" x 5⅜" x 5".

MODEL 102 STANDARD

CLEAR PLASTIC CAGE FOR MICE. Has all the construction features described for Model 101 except the general shape is square instead of rectangular. Cage size for all 102 Models 11¼" x 11¼" x 6½".



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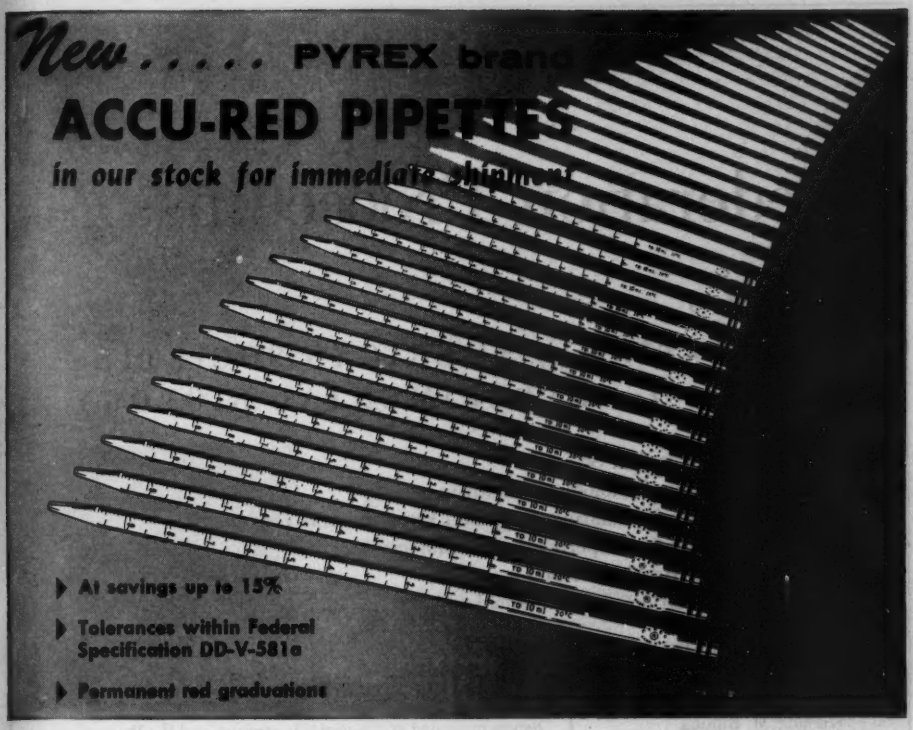
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Graduation interval, ml....	1/10	1/100	1/10	1/10	1/10	1/10
Tolerance, ±ml.	0.02	0.02	0.02	0.04	0.06	0.10
Each.....	1.03	1.14	1.16	1.16	1.34	1.81
Per original *package of 18.	16.69	18.47	18.79	18.79	21.71	29.32

8169-L. Pipettes, Serological, Accu-red, Pyrex brand glass, graduated to extreme tip; with two bands at top indicating they are calibrated for blowing out the last drop.

To deliver, ml.....	1	1	2	5	10
Graduation interval, ml.....	1/10	1/100	1/10	1/10	1/10
Tolerance, ±ml....	0.02	0.02	0.02	0.04	0.06
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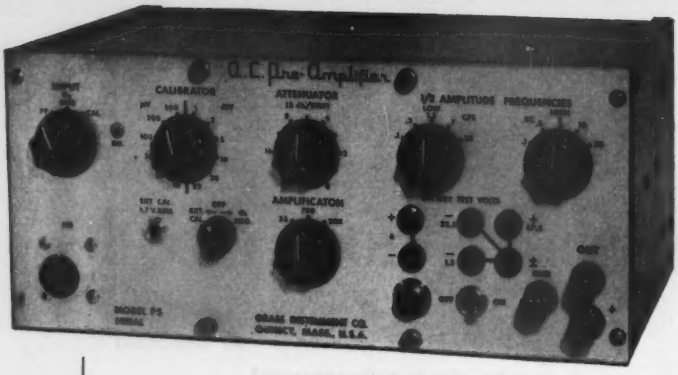
*The American Foundation: Medical Research: A Midcentury Survey, Boston, Little, Brown and Company, 1955, vol. 1, p. XXXI.

**Ibid., p. 600.



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Electron Micrograph showing segments of threadlike molecule of desoxyribose nucleic acid from calf thymus. Large particles are polystyrene spheres about 800 Angstroms in diameter. Judged from length of shadows, the nucleic acid molecule is about 20 Angstroms in dia. *Micrograph, courtesy Professor Cecil E. Hall, Associate Professor of Biology, Massachusetts Institute of Technology.*

Dr. Hall of M.I.T. was the first one to actually see and photograph a molecule of desoxyribose nucleic acid (DNA). This was made possible by his new and unique method of preparing specimens, which permits utilization of the greatly increased resolving power of the RCA EMU-3 Electron Microscope. The discovery is of considerable importance because it gives visual proof of the shape and size of these molecules—characteristics which otherwise only could be theorized.

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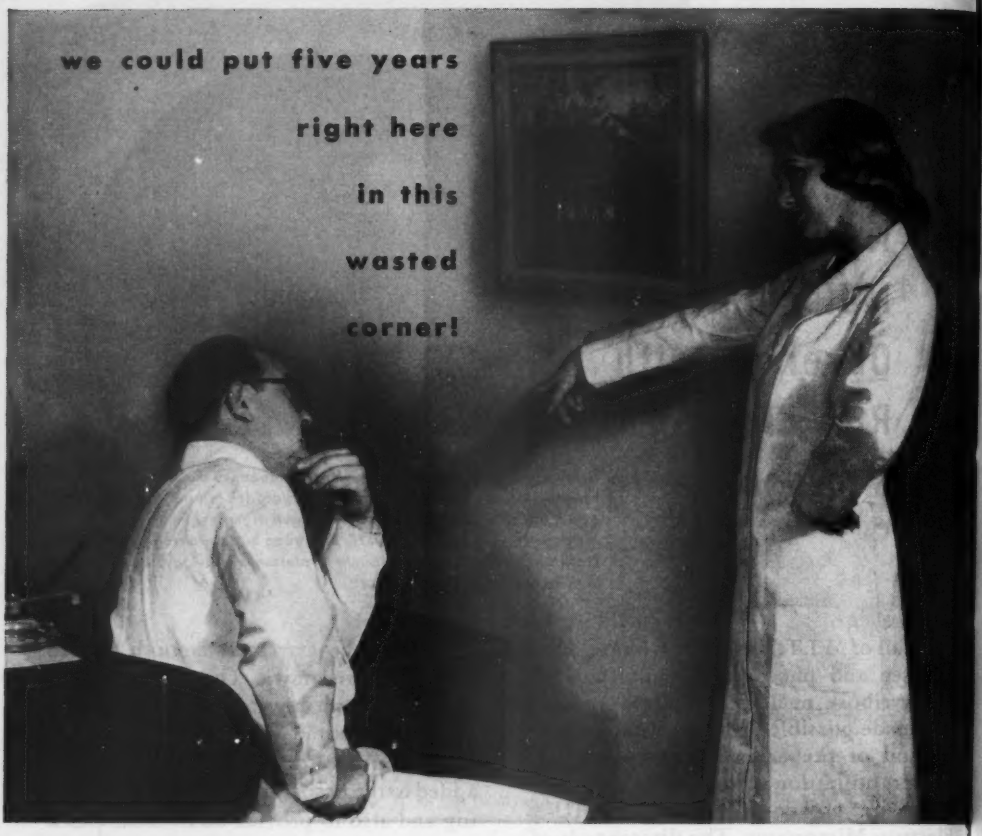

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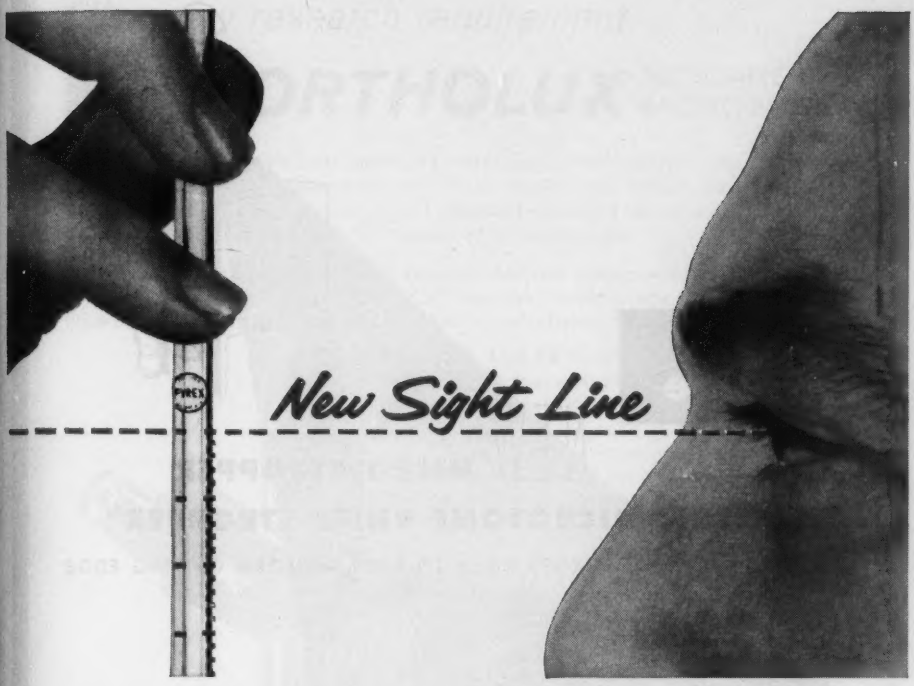

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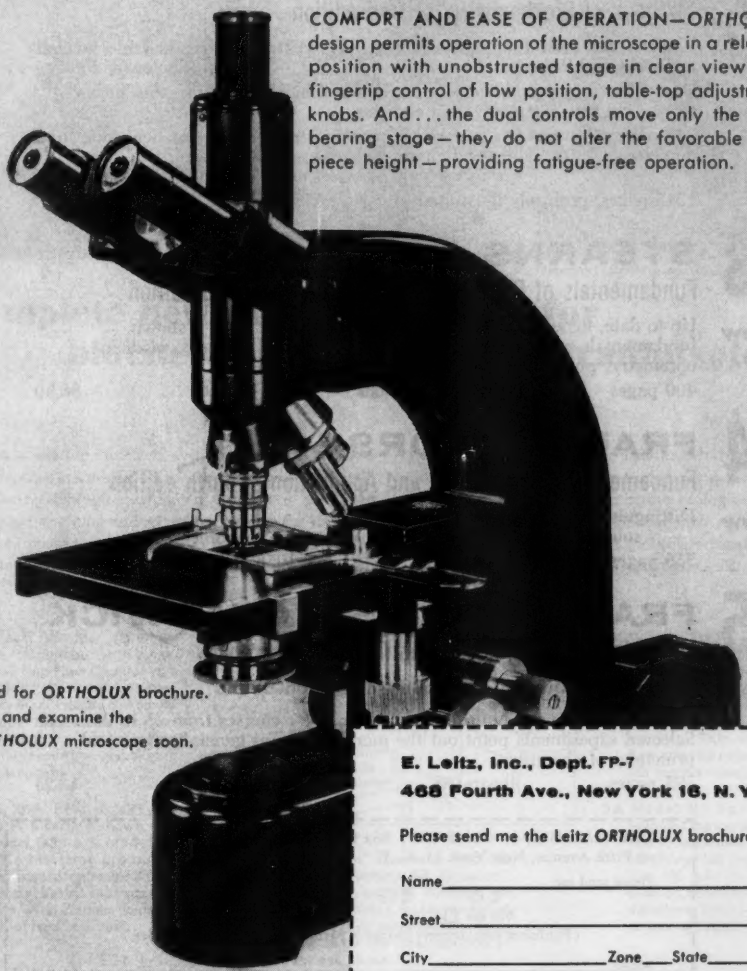

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(must be adjusted to patient's response)

To change from insulin to Orinase:

If previous insulin dosage was less than 40 u./day . . . reduce insulin 30% to 50% immediately; gradually reduce insulin dose if response to Orinase is observed.

more than 40 u./day . . . reduce insulin 20% immediately; carefully reduce insulin beyond this point if response to Orinase is observed. In these patients, hospitalization should be considered during the transition period.

Caution: During the initial "test" period (not more than 5 to 7 days), the patient should test his urine for sugar and ketone bodies three times daily and report to his physician daily. For the first month, he should report at least once weekly for physical examination, blood sugar determination, and white cell count (with differential count, if indicated). After the first month, the patient should be seen at least once a month, and the above studies carried out.

It is especially important that the patient, because of the simplicity and ease of administration of Orinase, does not develop a careless attitude ("cheating" on his diet, for example) which may result in serious consequences and failure of treatment.

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Indications. Orinase is most likely to benefit the patient in whom the diabetes is relatively mild and stable, is not adequately controlled by dietary restrictions alone, and developed sometime after the age of 30 years.

Contraindications. Orinase is contraindicated in patients with 1) diabetes of the type known variously as juvenile, growth-onset, unstable, or brittle; 2) a history of diabetic coma; 3) diabetes complicated by ketosis, acidosis, coma, fever, severe trauma, gangrene, Raynaud's disease, or serious impairment of renal or thyroid function; 4) hepatic dysfunction; and 5) diabetes adequately controlled by dietary restriction.

Effects. In patients with a satisfactory response to Orinase, the blood sugar falls, glycosuria diminishes, and such symptoms as pruritus, polyuria, and polyphagia disappear. It is *not* a substitute for insulin. And it requires the same adherence to basic principles of diabetes control as does insulin, e.g., dietary regulation; tests for glycosuria and ketonuria; hygiene; exercise; instruction of the patient to recognize and counteract impending hypoglycemia, to follow rigidly directions regarding diet and continuing use of the drug and to report immediately to the physician any feeling of illness. Extreme care must be taken during the transition period to avoid ketosis, acidosis, and coma.

Side effects. To date, the most serious side effect is hypoglycemia, which may occur occasionally and is most likely to occur during the transition period from insulin to Orinase. Other untoward reactions to Orinase are rare, usually of a nonserious nature, and tend to disappear on adjustment of dosage, e.g., gastrointestinal disturbances, headache, variable allergic skin manifestations, and alcohol intolerance.

Clinical toxicity. Aside from an occasional hypoglycemia, Orinase appears to be remarkably free of gross clinical toxicity. There is no evidence of crystalluria or other untoward effects on renal function, or of hepatotoxicity. Except for a rare leukopenia of mild degree, which has been reversible (in some instances, even under continued therapy), there have been no adverse effects on hematopoietic function.

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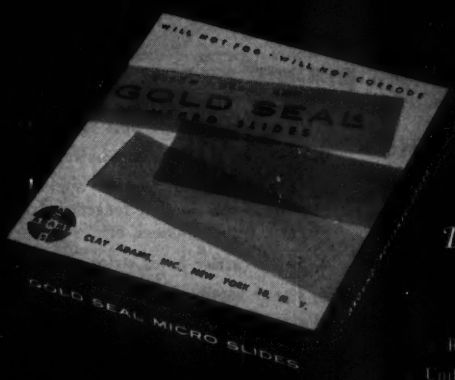

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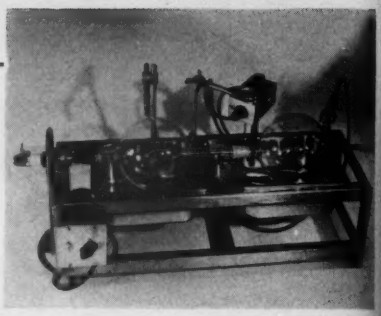

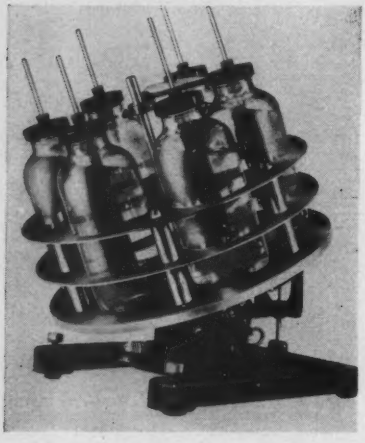

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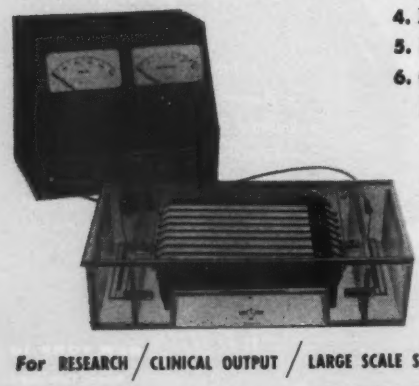

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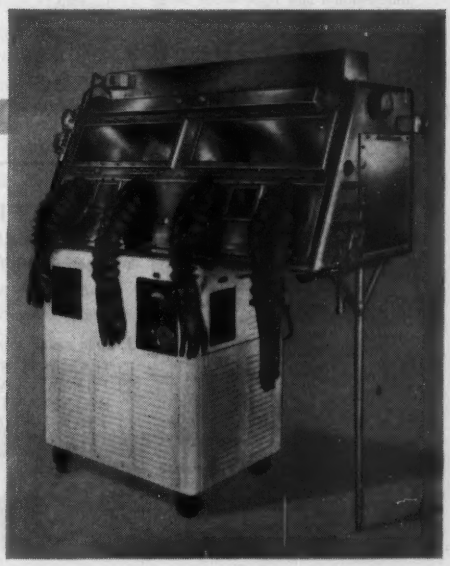

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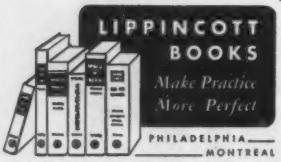

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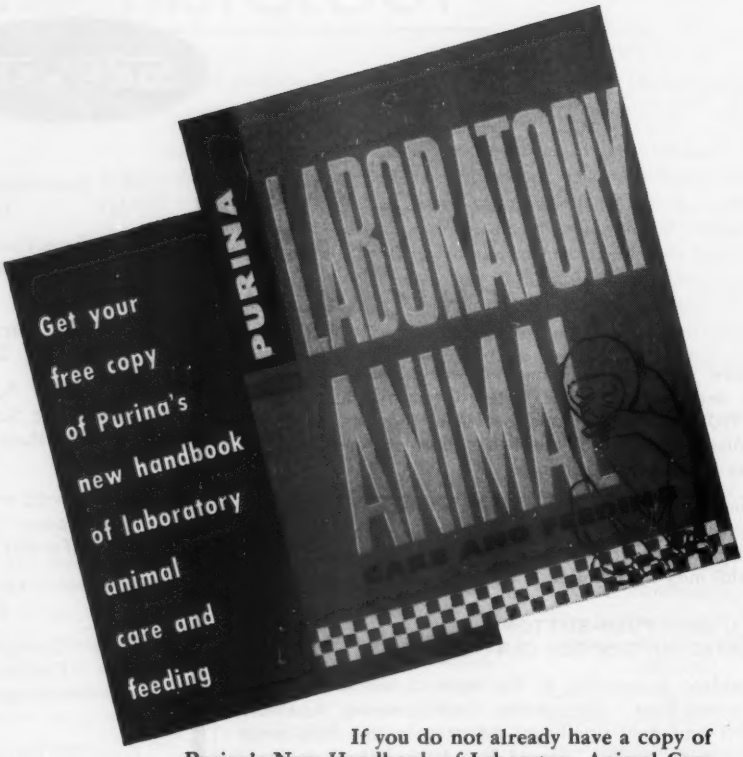

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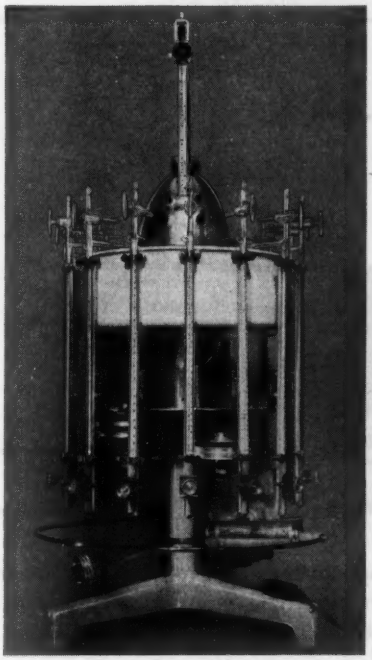

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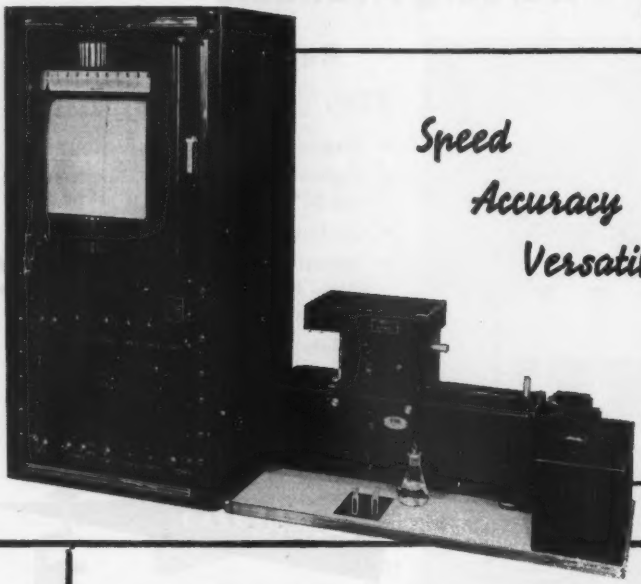

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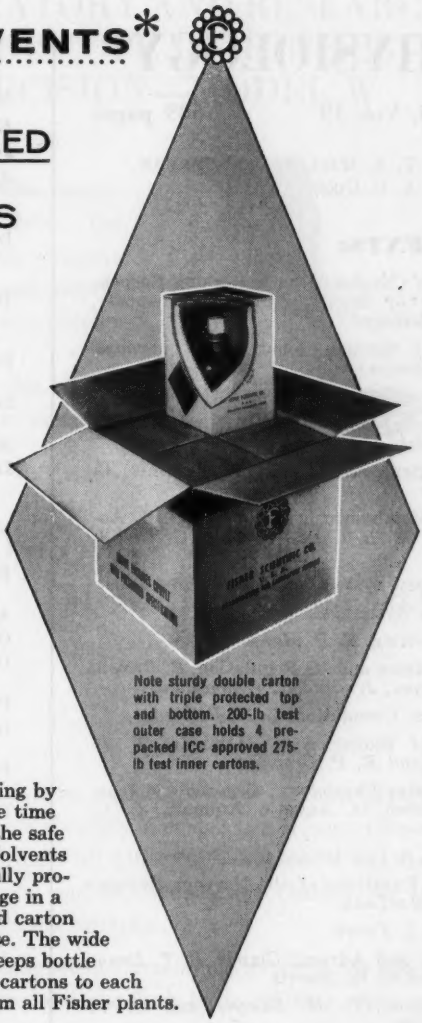

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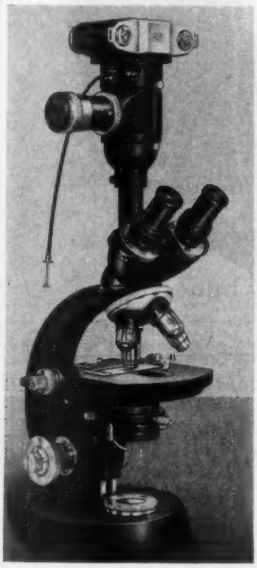

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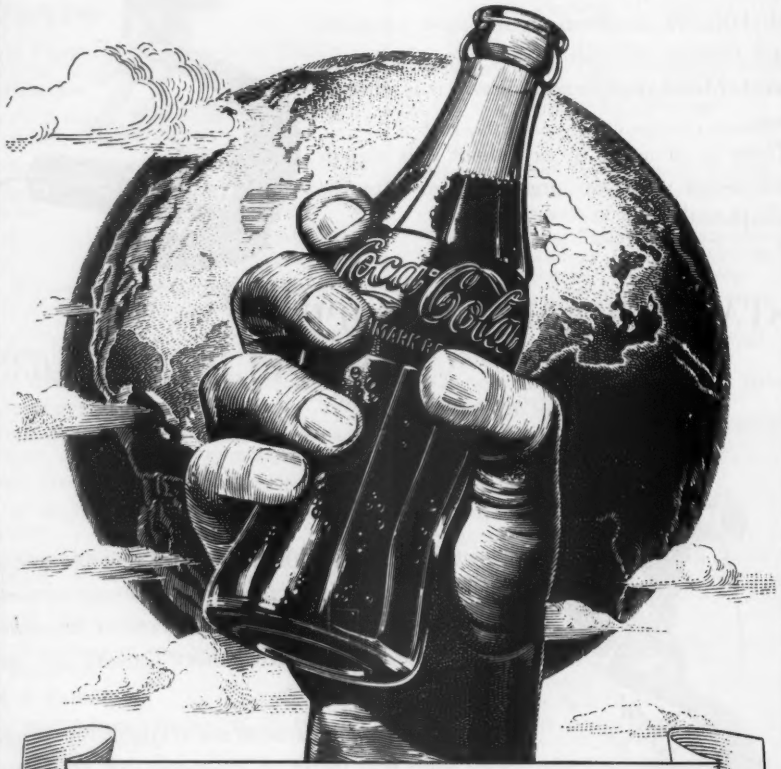

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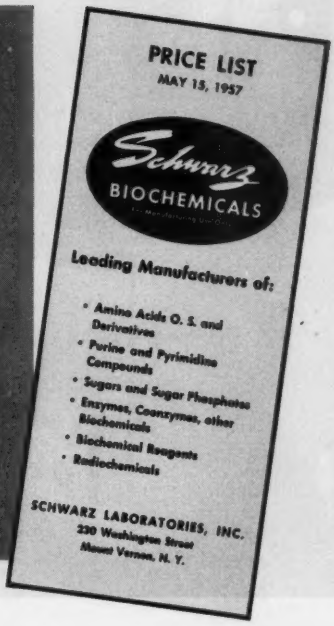

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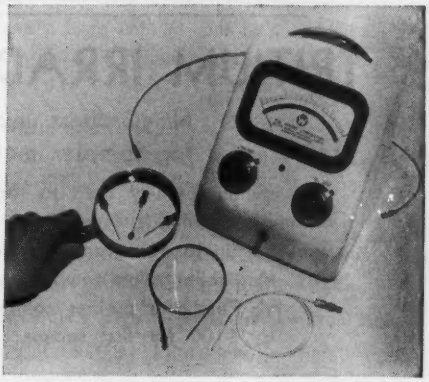

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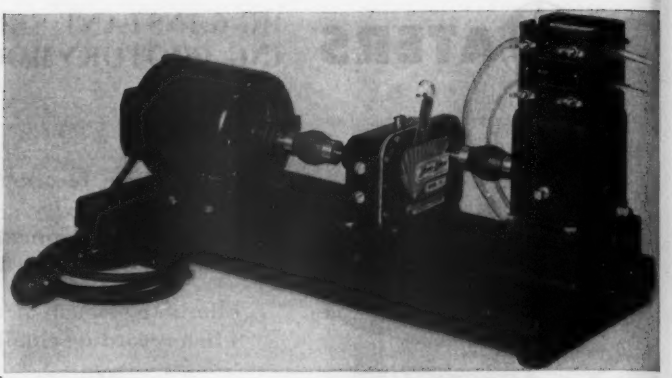

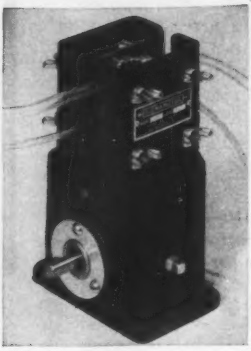

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*K. Wilzbach, J. Am. Chem. Soc. **79**, 1013 (1957).

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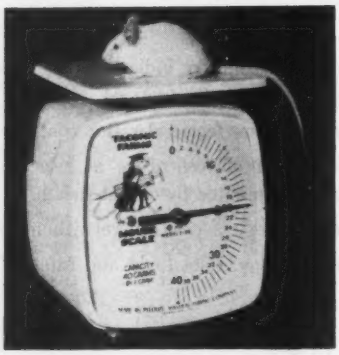

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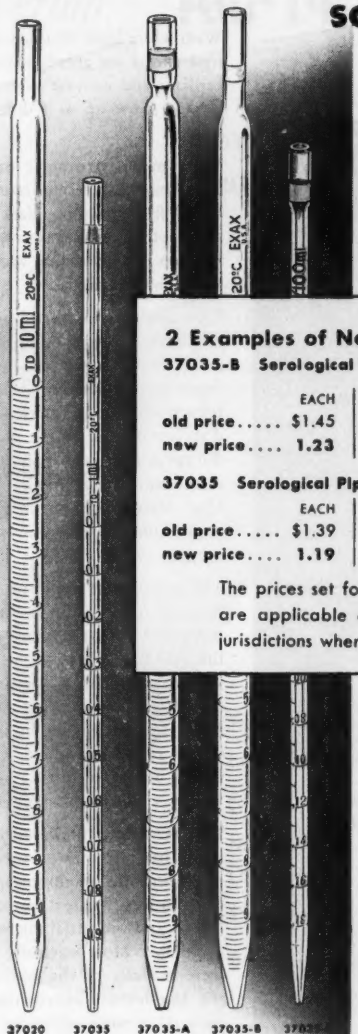

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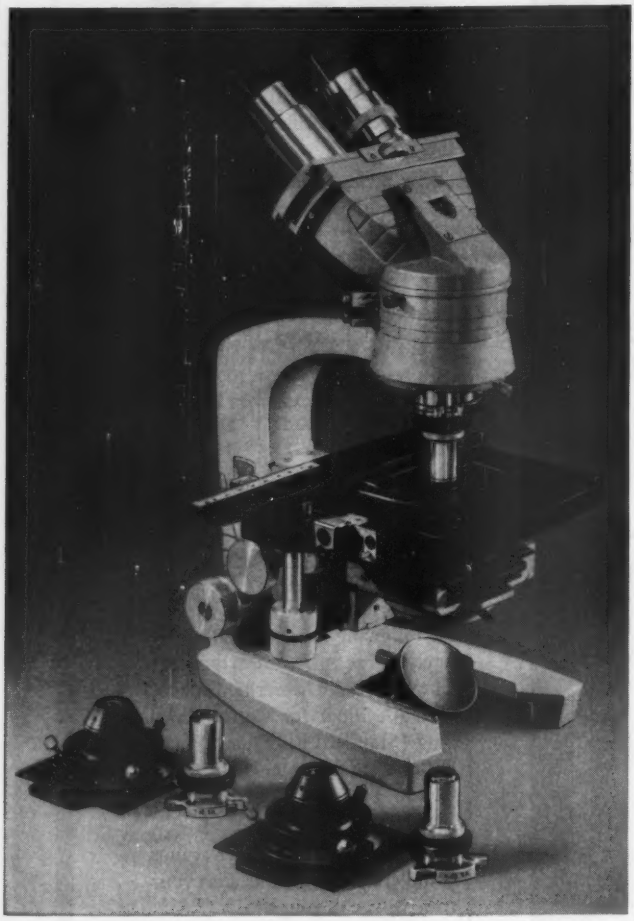

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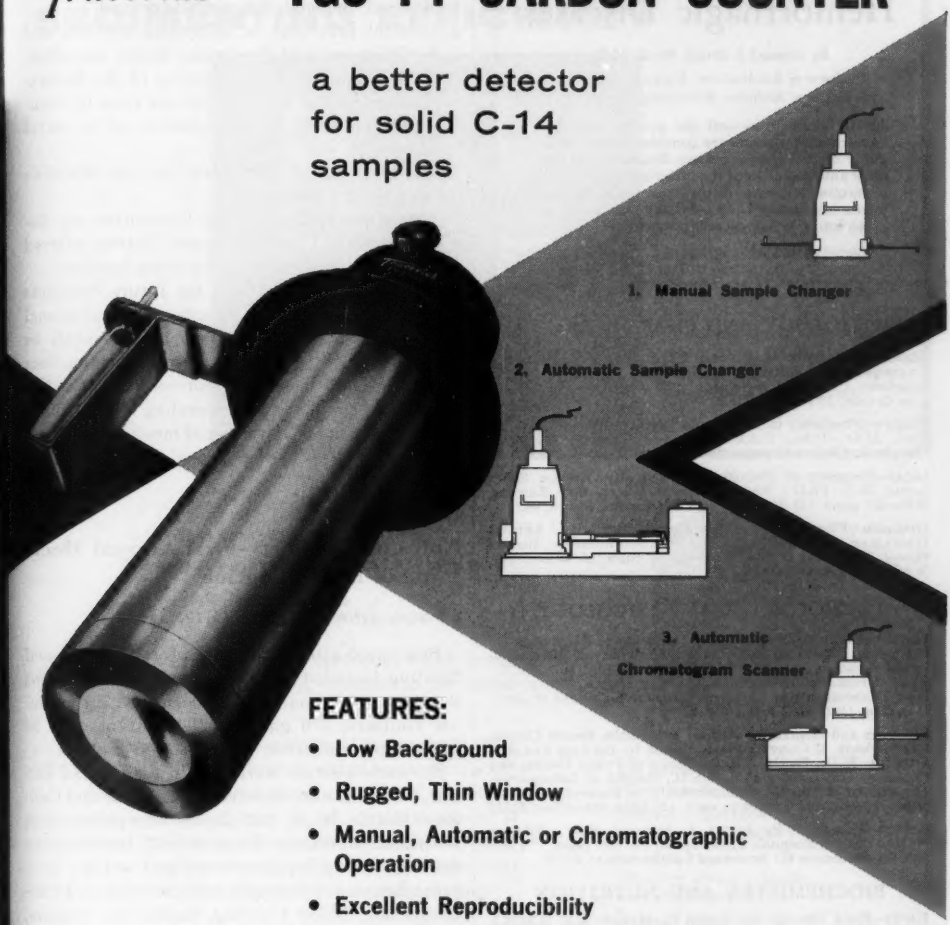

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News & Notes

Summary of Federation Board Meeting, April 15, 1957

1. Revised financial statement approved.
2. General principles of allocating services and facilities costs at Beaumont House approved.
3. It was voted that each Society of the Federation be granted option to use one room in Beaumont House for its headquarters at no rental charge.
4. Contribution of $250 voted to the Scientific Manpower Commission.
5. Recommendations of the Committee on Revision of the Constitution and Bylaws referred to the Society Councils for consideration.
6. Resolution adopted that for future contracts to administer travel awards to international meetings, the screening committees shall be appointed by the Board from nominations made by those societies represented on the National Committees corresponding to the Unions sponsoring the international meetings, in those instances in which National Committees do exist.

Notes on the Forty-First Annual Meeting of the Federation

Chicago, Illinois, April 15–19, 1957

Registered attendance at the forty-first Annual Meeting included 1,942 members of Constituent Societies, 4,022 non-member scientists, 794 graduate students, 379 guests, 521 exhibitors and 30 press representatives, a total of 7,688.

Scientific sessions were held in the Conrad Hilton, Palmer House, Sheraton-Blackstone and Congress Hotels. In all, 1945 papers were presented in 185 scientific sessions. There were 21 Inter-Society Sessions in which papers from each society were presented on the topics of Acetylcholine and Cholinesterase, Blood Clotting, Radiation, Tumors, Atherosclerosis and Connective Tissue. Society symposia presented during the week included the following: three in Physiology on 'Utilization of Recorded Knowledge', 'Tissue Oxygen Tension', 'Bioclimatology' (presented jointly with the Committee of Bioclimatology and the American Meteorological Society); three in Biochemistry on 'Structure and Function of Proteins', 'Chemistry and Metabolism of Phosphatides' and 'Molecular Heterogeneity of Hemoglobin'; one in Nutrition on 'Nutrition and Food Technology'; one in Immunology on 'Gram-Negative Bacteria.' One teaching session, a Physiology panel, discussing 'Graduate Students in Physiology' was presented.

(cont. on page xliv)

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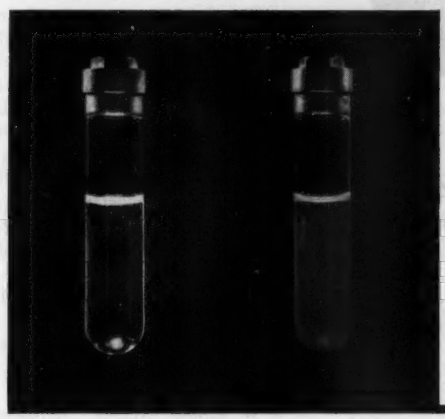

These two tubes containing 5 mg/ml of tobacco mosaic virus were centrifuged for 1 hour at 25,000 rpm, 51,000 x gravity. In tube at right, run in conventional rotor, 12% of virus remains in supernatant. In tube at left, run in K-25.1 Insulated, Core-Ventilated Model K rotor, only 2% remains in supernatant.

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The key: high centrifugal forces (to 51,000 x gravity) plus a unique insulated, core-ventilated* rotor. This new rotor minimizes thermal convection which causes stirring in conventional rotors; it keeps rotor temperatures from rising more than a few degrees above ambient; it protects most labile materials without requiring refrigeration.

The results: sedimenting efficiency is greatly increased; the time for centrifuging routine samples is substantially reduced; many separations are possible with the Model K which cannot be done with conventional centrifuges.

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The American Society for Artificial Internal Organs held scientific sessions and business meetings April 14 and 15 in conjunction with the Federation Meetings, and the Poultry Nutrition Group conducted a conference on April 14.

Meetings of Councils of the six member Societies and the Federation Board were held April 14 and 15, and new business was submitted at the Society Business Meetings April 16. In addition, Editorial Boards of the journals of the Member Societies, the Society for Experimental Biology and Medicine, the Society for Research on the Reticuloendothelial System, American Institute of Biological Sciences, the National Institutes of Health, as well as a number of special committees, used the facilities of the Federation for meetings. The Pathology, Nutrition and Pharmacology Societies held dinner meetings on Wednesday, April 17. The Biochemistry and Nutrition Societies sponsored a Smoker on Wednesday evening. Special interest and alumni groups met for breakfasts, luncheons, dinners and social gatherings throughout the week.

The American Association of Immunologists, host society at the Meeting, sponsored the Joint Session on Tuesday evening, April 16. Dr. Merrill W. Chase, Acting Chairman of the Federation Board, presided. The three papers on 'Immunological Tolerance,' presented by Dr. Ray D. Owen, Dr. Hilary Koprowski and Dr. Glenn H. Algire, appear in this issue of FEDERATION PROCEEDINGS.

A Ladies Committee headed by Mrs. Richard Winzler provided a Hospitality Lounge and greeted the wives of member and non-member scientists. An interesting program of activities was arranged, beginning with a visit to the Prudential Building, a tour to Argonne National Laboratories, a luncheon and Fashion Show at Marshall Field's and a visit to the University of Chicago. The Federation is indebted to the following committee for making the week a pleasant one: Mrs. Austin Brues, Mrs. Daniel L. Harris, Mrs. John Annegers, Mrs. Piero P. Foa and Mrs. Herbert Carter.

Dr. John O. Hutchens, with the assistance of Mr. Ralph Rohweder, conducted a tour of the Federation Meetings for high school teachers and their students from the Chicago area. The teachers and students were invited to visit the exhibits and meetings so that their understanding of the content and purpose of biological research could be increased by observing how scientists exchange information, formally in meetings and informally in groups outside sessions.

The Public Information Committee, headed by Dr. Richard Winzler, assisted by Drs. Alfred Gilman, I. M. Hoobler, Simon Rodbard, Edward L. Tatum, Byron H. Waksman and Robert W. Wissler scheduled press conferences and were available

(*cont. on page xlvi*)

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to assist members of the press with the scientific aspects of the papers they reported.

Books, apparatus and equipment, pharmaceuticals, chemicals and food products were among the 120 industrial exhibits on display in the Conrad Hilton; 6 institutional exhibits depicted the progress and activities of the societies represented, and 17 members displayed their work.

1958 Annual Meeting

The next annual meeting of the Federation will be in Philadelphia, April 14–18, 1958.

Placement Service

The Placement Service office in the Conrad Hilton Hotel during the meetings was a very busy place. Both candidate and employer registrations were unusually heavy during the first sessions open for them. Consequently, interviews were scheduled early in the week and on Tuesday, the first interview day, all interview booths were filled all day long, except for a few over the lunch hour. This indicates an increasing cooperation and interest on the part of all users. Next year a larger staff will be on hand the first day and it is hoped this will eliminate some of the delays experienced by early registrants in 1957, especially among the employers.

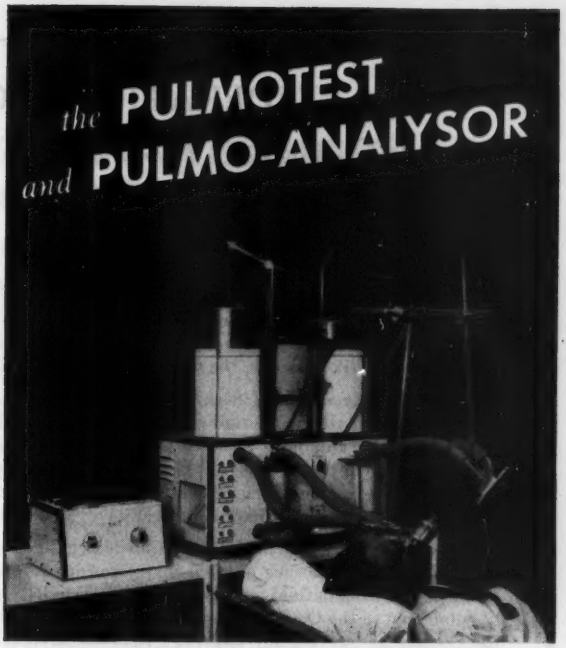
A total of 351 candidates registered. Of these, 170 indicated their chief field of training to be in biochemistry; 90, physiology; 45, pharmacology; 2, experimental pathology; 12, nutrition; and 32, immunology and/or microbiology. Of these, 295 or 84% received appointments. This percentage has been increasing the last three years, after remaining consistently at 66–68% for the previous years for which there are records.

The employers' desk registered 257 in 1957–107 of these, the first morning it was open—compared with 205 in 1956. Those who had notified the Washington office that they expected to use the interview facilities of their subscriptions found their credentials awaiting them. Those who had not done so experienced some delay until the credentials could be prepared or new subscriptions processed.

A total of 1398 interviews was arranged. Approximately 100 of these were canceled or did not appear at the scheduled time. Many of these individuals met or talked together earlier and agreed to forego the interview. However, the staff was aware that at least 100 more interviews took place in or near the Placement offices, arranged informally and concerning which there is no record.

Even though there was about the same number of candidates on the rolls in April 1957 as in April 1956, the May 1957 list of candidates is a little smaller than last year. In the four weeks succeeding the meeting an unusually large number of can-

(*cont. on page 662*)



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FEDERATION NOTICE

Membership in the Federation of American Societies for Experimental Biology

There is no provision in the Federation Constitution for individual membership. An individual wishing to associate himself with the Federation must become a member of one or more of the six constituent Societies; such membership entitles him to present papers at the annual meetings of the Federation and to receive the quarterly publication, FEDERATION PROCEEDINGS. Since requirements and procedures for election to membership vary among the six Societies, the following information is provided:

1. *American Physiological Society.* Any resident of North America who has conducted and published meritorious original researches in physiology shall be eligible for membership. Names of candidates must be proposed in writing by at least two members of the Society, and from these names the Council shall nominate candidates for election to membership at a regular meeting of the Society. *For further information and proposal forms, address* DR. R. G. DAGGS, Executive Secretary, 9650 Wisconsin Ave., Washington 14, D. C.

2. *American Society of Biological Chemists.* Qualified investigators who have conducted and published meritorious original investigations in biological chemistry are eligible for membership. Nominations shall be made and seconded by members of the Society and shall be submitted to the Council, who shall determine eligibility and make recommendations to the Society at any regular meeting. *Nomination blanks and further information may be obtained from* DR. PHILIP HANDLER, Duke University School of Medicine, Durham, N. C.

3. *American Society for Pharmacology and Experimental Therapeutics.* Any person who has conducted and published meritorious investigations in pharmacology or experimental therapeutics and who is an active investigator in one of those fields, shall be eligible to membership. Candidates shall be proposed by two members who are not members of the Society Council, for consideration and recommendation by the Membership Committee and the Council. *Further information and application forms may be obtained from* DR. H. C. HODGE, University of Rochester School of Medicine, Rochester, N. Y.

4. *American Society for Experimental Pathology.* Any American investigator who has contributed meritorious work in pathology within three years prior to his candidacy is eligible for membership. Candidates shall be nominated by two members and the nominations approved by the Council for election by Society members at a regular meeting. *Additional information and nomination forms may be obtained from* DR. J. F. A. MCMANUS, Medical College of Alabama, Birmingham, Ala.

5. *American Institute of Nutrition.* Qualified investigators who have independently conducted and published meritorious original investigations in some phase of the chemistry or physiology of nutrition, and who have shown a professional interest for at least five years, are eligible for membership. Nominations shall be made and seconded by members of the Society and shall be submitted to the Council for recommendation to the Society at any regular meeting. *For nomination blanks and additional information address* DR. G. M. BRIGGS, National Institutes of Health, Bethesda, Md.

6. *American Association of Immunologists.* Any qualified person engaged in the study of problems related to the purpose of the Association may apply for membership. Candidates must be nominated by two members of the Association and applications must be accompanied by letters of recommendation, curriculum vitae and a list of reprints of publications. The Council shall determine eligibility and submit the names of eligible candidates to the membership at the annual meeting. *For further information and application blanks, address* DR. F. S. CHEEVER, Secretary, University of Pittsburgh, Graduate School of Public Health, Pittsburgh, Pa.

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Federation Proceedings

VOLUME 16 *July 1957* NUMBER 2

JOINT SESSION OF THE FEDERATION[1]

Immunological Tolerance

Chairman: MERRILL W. CHASE

IMMUNOLOGICAL TOLERANCE

RAY D. OWEN

From the California Institute of Technology, Pasadena, California

THE PURPOSE of this Session is to share with a diverse audience certain developments that are currently exciting the interest of immunologists. The field of 'immunological tolerance' has developed explosively within the past few years. Our aim is to cover what appear at present to be salient points and key references in this area. In the following paper, Dr. Koprowski will elaborate the studies of transplantation of normal tissues and tumors that have contributed in very large part to the development of this field. I will introduce this part of the subject very briefly, but shall spend most of my time in diversions to similar effects of non-viable antigenic materials. In the concluding paper, Dr. Algire will illustrate techniques that have increased our understanding of immune reactions to foreign tissues.

Spontaneous Exchange of Erythropoietic Tissues Between Twin Embryos. An early indication of what later was to be developed into the concept of immunological tolerance was provided by one of Nature's experiments—the exchange of materials between twin embryos in cattle (1). It had been noted, by members of the Wisconsin group, that twin cattle showed identical blood types much more often than should have been expected from estimates of the frequency of identical twinning. An indication of the basis of this concordance was provided by a pair of twins that had different sires (table 1). The twins had identical blood types, and in terms of this type neither of the twins could have been produced by either of the possible sires and the dam. Certain reagents that usually yield complete hemolysis of positive test cells caused only an incomplete lysis of the red cell suspension from each twin. When the cell suspension of either twin was lysed by complement after sensitizing with the R reagent, and the unaffected cells were recovered and washed, it was found that these cells not only lacked the R specificity, but G and I′ as well. On the other hand, they were *all* lysed by the S and X_2 reagents. Similarly, lysing the initial cell suspensions with the X_2 reagent removed SX_2 cells, but left GRI′ cells unaffected. The circulating red cell population of each twin, therefore, was composed of a mixture of two distinct cell types, one of which was derived from the zygote formed when a Hereford (RI′) sperm fertilized an egg carrying an allele controlling the cellular specificity G, and the other when a Guernsey sperm (SX_2) fertilized another egg lacking that allele.

In view of the long-known placental anastomosis of blood vessels in bovine twins, it might not be considered surprising to find that the twins showed blood admixtures. However, these twins were almost a year old when they were first tested, and tests at intervals for more than an additional year continued to show the same phenomenon. It was evident, therefore, that the effective exchange between the embryos had been of potentially hematopoietic cells, which had undergone natural transplantation between the co-twins. That the mature hosts continued

[1] Chicago, Illinois, April 16, 1957.

TABLE 1. REACTIONS OF RED CELL SUSPENSIONS IN A CASE OF SUPERFECUNDATION

Cells	Tested for				
	G	R	S	X_2	I'
Untreated					
Guernsey sire	0	0	4	4	0
Hereford sire	0	4	0	0	4
Dam	4	0	0	0	0
Guernsey twin	0+	tr	2	2	tr
Hereford twin	tr	tr	1	1	tr
*After lysis with 'R' reagent**					
Guernsey sire	0	0	4	4	0
Hereford sire			no cells		
Dam	4	0	0	0	0
Guernsey twin	0	0	4	4	0
Hereford twin	0	0	4	4	0
*After lysis with 'X_2' reagent**					
Guernsey sire			no cells		
Hereford sire	0	4	0	0	4
Dam	4	0	0	0	0
Guernsey twin	4	4	0	0	4
Hereford twin	4	4	0	0	4

* Tests on washed, intact cells recovered after specific hemolysis.

0 = no lysis; 0+, tr, 1, 2 = increasing degrees of lysis; 4 = complete lysis.

to tolerate these transplants called attention to a fact long known to experimental embryologists: tissues grafted from one embryo to another often survive far beyond the period when incompatibility reactions should have developed to destroy them (cf. 2). In fact, the frequent survival of skin transplants between even newly hatched birds (the 'Danforth preparation', ref. 3) has provided opportunities for fruitful study (4). Together with the observations of Traub (5), the twin cattle observations provided the main factual cornerstone for the development, by Burnet and Fenner (6), of a theory of immunological tolerance.

We collected data on approximately one hundred additional pairs of cattle twins, of ages up to eight years, and on higher multiple births—triplets, quadruplets and a set of quintuplets (7). In twins, it was found that the two cell types generally occurred in approximately equal numbers in the circulation of each twin, the proportion varying in different twin pairs from 50–50 to 40–60, with a few pairs showing somewhat more divergent ratios. In cases in which one cell type was predominant, the same cell type was generally found to be predominant in both twins, a clear indication that the foreign cells were not at a selective disadvantage. The evidence showed that the cells were established and retained as intact units, developing autonomously as a function of their own genotype; there was never any indication that exchanges of determinants at the sub-cellular level had produced 'transformation' for some of the gene-controlled antigens in which the members of a twin pair differed, and not of others. One antigen, J, however, proved to be characteristic of the host body, and to be found on all of his red cells or absent from all of them as a function of the host genotype regardless of the genetic constitution of the cells (8).

Carr (9) noted that placental fusion sometimes occurs in mice and rats, and suggested that a genetic defect in one member of a litter may be remedied by the spontaneous transfer of normal tissue from another in such instances. Situations similar in detail to the twin cattle phenomenon have been described in sheep by Stormont, Weir and Lane (10), and in man by Dunsford and associates (11). Here, a woman designated as *Mrs. McK* was shown to have in her circulation red cells descended from her co-twin. This twin had died as an infant, but it was possible to determine his blood type rather completely through studies of descendants of his cells surviving in his sister's circulation more than two decades after his death. Ripley (12) achieved an experimental copy of the twin cattle situation by injecting embryonic rat cells intravenously into other rat embryos, and Hasek (13) produced a similar situation by artificial parabiosis of embryonated hens' eggs. The latter situation was defined by Billingham, Brent and Medawar (14), who applied the techniques of differential hemolysis described above to the demonstration of erythrocyte chimerism in chicks. Heterospecific egg-to-egg parabiosis has also been studied (13, 14).

Tolerance of Skin Transplants Conferred by Injecting Viable Tissue Cells into Embryos. The elegant experimental definition of immunological tolerance by Medawar and his group will be reviewed by Dr. Koprowski in the following paper. The first important extension was the demonstration that twin cattle commonly accept skin transplants from their co-twins (15, 16). [More recently, a successful kidney transplant has been achieved (17)]. In the classic paper that defined and established the field of immunological tolerance (18), Billingham, Brent and Medawar showed that the simple injection of living cell suspensions into embryos conferred a

persistent tolerance to skin transplants from the donor strain. We will defer to Dr. Koprowski's paper the further description of this part of our subject. At this point, we may recognize that the establishment of foreign cells accepted as transplants by an embryo confers a persistent tolerance toward tissues of the donor specificity. We may now ask whether the tolerance-conferring antigenic material needs to consist of viable and perpetuated donor cells, or whether non-surviving antigens introduced into embryos or very young individuals may also induce a depression of later immune responses.

Depression of Immune Responses to Red Cells. The erythrocytes of birds and mammals have a circumscribed life span; there can be little question that these cells disappear from the system, at least as intact cells, over an interval after their injection. Red cells also provide some advantages for studies of circulating antibodies, because serological test systems for red cell antigens are sensitive and easily conducted. Accordingly, injections of red cells into embryos or new-born or newly-hatched mammals and birds may be expected to provide a test of the necessity of cell survival for the maintenance of a state of persistent immunological tolerance.

Burnet, Stone and Edney (19) included human blood cells among the antigens used in early tests of a theory of antibody formation and tolerance, but observed no effects on the development of normal antibodies against human red cells in birds that had been treated with this same material as embryos. Billingham, Brent and Medawar (20), however, found that injection of chicks at hatching with adult chicken red cells resulted in a marked depression of the antibody response to later injection of the homologous material. That such depressions are not restricted to intraspecific combinations is indicated by the observation of Hasek (13), that the injection of goose blood into duck embryos resulted in an obvious suppression of later antibody formation, although no such suppression was observed when turkey blood was injected into chick embryos, guinea fowl into chick, duck into chick, chick into duck, duck into goose or chick into guinea fowl. Simonsen (21), however, did note a significant suppression of later agglutinin formation when turkey blood was injected into chick embryos. We have found (fig. 1) a rather consistent depression of the level of normal antibody development to human O

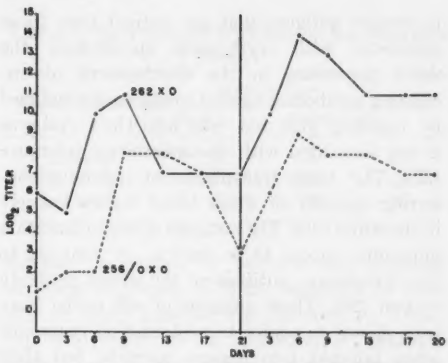

FIG. 1. Log_2 titer values against human O blood cells for serum samples from a control chicken (262) and one that had been injected as a day-old chick with human O blood (256). Both birds were injected with the human O blood when they were 3 months old (day 0 on the graph), and again 21 days later (22).

blood, and a delay and reduction in the 'primary' agglutinin response to this material, among chicks that had been injected within a day after hatching with a particular human O blood (22). We have not observed such effects with human AB blood. A provocative possibility is that the specific potentiality for 'normal antibody' production on the part of the host may play a part in such effects. Somewhat similar delays in the agglutinating antibody response were also observed in rats and rabbits injected neonatally with the human O blood. Simonsen (23) also observed effects produced by injecting human O blood into chicks, and noted that the depressing effects of injections are greatest when the embryo is in the 17th to the 19th day of development; this period appears to be preceded by one in which intraembryonic injections have less effect, and the sensitivity again decreases after hatching.

The relationships between this partial depression of circulating antibody formation to erythrocytes, and the field of tolerance to tissue transplantation, may be debated. The following points should be kept in mind: tissue transplantation immunity seems to depend in large part on a different channel of immune response than the development of circulating serum antibody (cf. 24). It appears to be analogous to delayed hypersensitivity, cell-borne and cell-mediated, a system in which no necessary cause-and-effect dependence on circulating serum antibody has yet been established (25). In tissue transplantation immunity, the channel of immune response appears

to involve antigens that are distinct from those associated with erythrocyte specificities; the sharp depression in the development of circulating antibodies against erythrocytes induced by injecting pure red cells into chick embryos is not associated with transplantation tolerance (20). The tissue-transplantation tolerance-conferring capacity of whole blood resides entirely in the white cells. The antigens of transplantation immunity appear to be nuclear, in contrast to the cytoplasmic antigens of the serum antibody system (26). These antigens of cell nuclei have been shown to confer transplantation immunity when injected into mature animals, but they have as yet failed to confer transplantation tolerance when injected into embryos or young animals in the absence of intact donor cells (27).

The injection of red cells into young birds and mammals brings about only an incomplete depression of later antibody responses, or it seems sometimes to produce delays in responses that may be confused with total suppression. There have been indications that an apparent depression sometimes derives in part from another kind of channelization of the immune response, namely, into the development of incomplete rather than complete antibody (22). This suggestion, however, has not been confirmed; it is possible that it depends on the particular route, time and quantity of injection of the chick, or perhaps on the techniques used to test for the presence of incomplete antibody. Furthermore, the duration of the period in which a partial depression of antibody responses is displayed has not been thoroughly investigated in connection with injections of erythrocytes. Such data as are at hand on the effects of injecting red cells (e.g. 23) indicate that the partial suppression of the circulating antibody response, at least when interspecific combinations are involved, is transient. Bauer, Peckham and Osler (28) observed only a slight and insignificant depression of hemolysin formation when 15-day fetal rats injected with sheep red cells were again injected at 9–10 weeks of age; significant depression was noted in rats that had been injected shortly after birth. The hemolytic system would probably detect incomplete as well as complete antibody, and the test age of 9–10 weeks may have passed the period in which depression may have been significant. A simple delay in response, of the sort we have noted in rats, might have been missed in Bauer's procedure of testing a single bleeding six days after the 'immunizing' injection. Ambrus and associates (29) observed no depression of response in chicks that had been injected with rabbit red cells as 6-day embryos; this observation is consistent with Simonsen's recognition of an early insensitive period. There are therefore conspicuous differences between the effects of erythrocyte injections and the control system of transplantation tolerance.

Possibility of Maternally Conferred Tolerance to Rh Antigens in Man. When an Rh-positive woman is carrying an Rh-negative fetus, it is possible that maternal cells or antigenic material may sometimes get across the placenta into the fetal circulation. If the Rh-negative fetus acquired a degree of tolerance toward this foreign antigen, such an individual might be expected to display a delayed, reduced or modified response upon exposure to Rh antigens in later life, in contrast to the responses of Rh-negative individuals whose mothers were also Rh-negative and who could not therefore have been exposed as embryos to the Rh antigens. Some years ago we undertook to test this possibility (30). Rh-negative women were classed as 'intolerant' of the Rh antigen if they produced circulating Rh antibodies in a third or earlier Rh-positive pregnancy; they were classed as relatively 'tolerant' if no antibodies were detected under these conditions. The data indicated (table 2) a statistically significant relationship between the 'tolerance' of the Rh-negative women and their mothers' Rh types. The daughters of Rh-negative women more frequently develop Rh antibody at an early opportunity. It should be emphasized that this apparent effect on the development of antibody of the sort tested in this study is not reflected in a differential frequency of erythroblastotic babies born to the Rh-negative daughters of Rh-negative women compared with the Rh-negative daughters of Rh-positive women. Our data confirmed the prior findings of Booth and associates (31), recently further confirmed by Ward, Walsh and Kooptzoff (32), that if one begins with a population of women who have erythroblastotic babies one does not find a differential incidence of Rh-negative as compared with Rh-positive maternal grandmothers of these babies. If our limited and preliminary studies may be accepted as providing an indication of a depression or delay of agglutinating antibody response as a function of 'maternally conferred tolerance,' a population situation somewhat as follows is suggested: the Rh-negative daughters of Rh-positive women

may commonly enjoy a degree of tolerance to the Rh antigen in terms of a reduced and/or delayed development of complete antibodies in response to repeated exposures to fetal Rh antigens. They are not, however, prevented from giving the kind of antibody response that is important in the development of erythroblastosis in their babies, a possibility that finds some support in our experimental work with chicks, mentioned above. It is possible that there may be a more subtle kind of compensation involved in such data. There may be some tolerance, even by the criterion of erythroblastosis, in some Rh-negative daughters of Rh-positive women, but in population data this may be compensated by a greater tendency on the part of other Rh-negative daughters of Rh-positive women to produce the sort of antibody important in erythroblastosis.

These indications of maternally conferred tolerance to Rh antigens are very tentative at present, and a definitive test of the possibility would be most welcome. A competent test depends on a careful experimental design. A group of Rh-negative women, married to Rh-positive men, must have their sera checked for Rh antibodies in successive pregnancies, whether or not any evidence of erythroblastotic sensitization appears. Data on women who do *not* develop antibody in an early Rh-positive pregnancy, and attention to antibody development as a function of number and sequence of exposures, are essential parts of a proper test design. Dependence on appearance of erythroblastotic children for the selection of women whose mothers are to be checked for their Rh type cannot provide evidence for or against the possibility.

Billingham, Brent and Medawar (14) have indicated that "a naturally acquired tolerance of maternal homografts is believed to occur very rarely in guinea pigs, presumably by the accidental incorporation into a fetus of maternal cells. No such natural tolerance has been observed in mice or rabbits." These authors have also called attention to a similar possibility in connection with maternal-to-fetal transfer of certain melanomatous tumor cells in man. The relationship between the mammalian mother and fetus, with regard to the phenomena of tolerance, has been further discussed by Medawar (33) and by Brambell (34).

Effects of Prenatal and Neonatal Exposure to Viruses and Bacteria. Several studies have suggested that embryonic and/or neonatal exposure to viruses or bacteria may suppress antibody responses to these materials in later life. An early example was provided by Traub (5), who observed an inhibition of antibody production to lymphocytic choriomeningitis virus in mice that had undergone prenatal exposure to this virus. Buxton (35) obtained no antibody response to killed *Salmonella pullorum* in chickens that had been exposed to this organism as embryos or young chicks, and Kerr and Robertson (36) noted a severe reduction in ability to produce antibody to *Trichomonas foetus* among cattle that had been injected with this antigen in early postnatal life. Related aspects of this subject will be discussed by Dr. Koprowski.

TABLE 2. RH TYPES OF MOTHERS OF RH-NEGATIVE WOMEN

Woman's Classification	Mother's Type		Total
	Positive	Negative	
Tolerant	32	9	41
Intolerant	27	29	56
Total	59	38	97

Depression of Immune Responses to Soluble Antigens. Dixon and Maurer (37) injected rabbits six times weekly for about one hundred days post birth with bovine albumin or human plasma. When the rabbits were again injected with these antigens at 10–11 months of age, they proved specifically unresponsive to the antigen with which they had earlier been injected. Adult animals given similar massive injections over long periods of time also showed a depression of antibody response, but this persisted only for as long as the injected protein remained detectable, a period of 3–4 months. Hanan and Oyama (38) injected alum-precipitated bovine serum albumin into pregnant rabbits, and into some of the offspring three times a week for 3½ months. The treated rabbits, when mature, were found not to develop antibody to bovine serum albumin. Their response to egg albumin appeared also to be affected—a result compatible with Maurer's report of cross-reaction between antiserum to egg albumin and bovine serum albumin.

Cinader and Dubert (39, 40) reported a specific inhibition of response to human albumin among rabbits that had been injected from 9 to 19 days after birth with total dosages over these intervals ranging from 20 to 1110 mg of the protein. Antibody responses after injections at 9–10 weeks of age and at intervals thereafter were checked by

a variety of techniques: agar diffusion methods, the agglutination of sensitized tanned erythrocytes, and elimination rates of antigen labelled with radioactive iodine from the circulation of the test animals. Controls consisting of untreated litter mates of the experimental animals regularly showed an antigen elimination curve characteristic of immune animals, and after several courses of injection during adult life their sera agglutinated tanned sensitized erythrocytes to titers ranging from 1/2000 to 1/50,000. The sera of animals given neonatal injections of the human albumin did not agglutinate tanned sensitized erythrocytes even at a dilution of 1/2, and their antigen elimination curves did not show the phase of rapid elimination characteristic of antibody development. These rabbits did not give evidence of antibody during the period of more than 600 days covered by the experiment.

Six of the rabbits with 'acquired tolerance' to human albumin were injected with a modified albumin diazotized with *p*-amino benzene sulphonic acid (DHA). Two of the six animals did respond to the injection of DHA, and their sera were able to agglutinate albumin-sensitized cells as well as DHA-sensitized cells. Furthermore, one of these two rabbits was later found to have lost its tolerance to the human albumin after his response to the modified albumin. It is, of course, possible that this was simply a spontaneous loss of tolerance. It is, however, evident that the modified albumin, recognized as foreign by the injected animal, evoked the formation of some antibody, capable of cross-reacting with the unmodified antigen. It is possible that this antibody constituted, or was causally associated with the constitution of, a 'recognition mechanism' through which human albumin itself could be treated as a foreign and antigenic material.

Smith and Bridges (41) injected crystalline bovine serum albumin into new-born rabbits, in dosages ranging from 1 to 200 mg protein. Reinjected at 4 and 8 months of age, none of the rabbits given a neonatal dosage of 20 mg or more responded by elaborating antibodies. Those that had been given neonatal dosages less than 20 mg gave variable responses and some indications of 'partial tolerance.' Cohn, however, injected various antigens into 14-day-old chick embryos and found no depression in the immune response upon challenging the chicks at 2 months of age (42). In view of the observations of Simonsen noted above, it is suggested that Cohn tested an embryonic stage prior to the most sensitive period.

One of the most extensive and incisive investigations of the depression of immune responses to soluble proteins is that of Wolfe and associates (43), who report effects of massive injections of bovine serum albumin at hatching on subsequent antibody production in the chick. These authors note that chicks injected at hatching show a marked and highly significant depression of antibody response when they are again injected at 6 weeks of age. At 12 weeks of age, certain experimental groups continue to show highly significant differences from the uninjected controls while others do not. By 22 weeks of age, the experimental and control groups give very similar responses. These studies of the chick, therefore, suggest a more transient depression of the immune response than has been reported for the rabbit, upon the injection of soluble antigens into the neonatal individual. Another difference is in the clearance time of injected antigen from the circulation of non-responding chicks injected at hatching, as compared with controls. The authors consider the possibility that their 'tolerant' chicks are actually producing non-precipitating antibodies, which effect antigen clearance from the circulation but are undetected in precipitin tests. They have, however, been unable to obtain direct evidence of such antibody.

Reference should also be made to the use of less well-defined mixtures of soluble antigens in 'tolerance' studies. Downe (44) reported that pre- and postnatal treatment of rabbits with whole chicken serum rendered them unable to develop precipitins for chicken serum in later life. Injected with turkey serum, these animals produced antisera lacking the antibodies cross-reactive with chicken serum generally characteristic of rabbit-anti-turkey sera. Hasek (13) found that precipitin production in mature chickens was not significantly affected if the chicks had been injected as embryos with turkey, guinea fowl or duck blood. Similarly, turkeys that had been subjected to egg-to-egg parabiosis with chicks, upon immunization at the age of 6 months, produced precipitins for chick serum indistinguishable from those of control turkeys. Hens that had as embryos been parabiotic with turkeys, however, showed a significant suppression of precipitin formation to turkey serum proteins. One chick that had been in parabiosis with a turkey as an embryo, and had been for a period a chimera of turkey and chicken cells,

had turkey serum proteins in the circulation during the period when turkey cells were also present. Later, both the turkey cells and serum proteins disappeared from the blood, but precipitins were not formed on immunization of this chick with turkey serum six months later.

It is therefore evident that phenomena at least grossly comparable to tissue transplantation tolerance can be observed with soluble antigens. The mechanisms through which they act may, however, be diverse, and distinct from those involved in actively acquired tolerance to tissue transplants conferred by the injection of living homologous cells into embryos or neonatal individuals. We shall now attempt to list briefly some of the procedures that, applied to adults, produce effects similar in one way or another to 'acquired tolerance.'

Inhibition of Delayed Drug Hypersensitivity by Feeding. Chase (45, 46) has defined and studied in detail a system that shows certain marked similarities to acquired tolerance. Briefly, the system involves the delayed skin reactions produced in guinea pigs by certain chemicals. The channel of immune response here is that of cell-borne and cell-mediated reactions. Chase discovered that when guinea pigs were fed one of these chemicals, a very persistent and specific refractoriness to skin sensitization was produced. No serum antibodies could be demonstrated, but the normal reactivity of the treated guinea pigs could be restored by injecting cells from peritoneal exudates of actively immunized guinea pigs. Here, therefore, as in actively acquired tolerance to tissue transplants, a specific inhibition of the central cellular response appears to be involved. Whether the mechanism of this effect relates in any specific way to the core phenomena of immunological tolerance remains to be determined.

Desensitization in Immediate Hypersensitivity. We would be led far afield, I fear, if we were to attempt to sample and evaluate the evidences of diminished immunological reactivity reported in connection with desensitization therapy in allergy (e.g. 47). Somewhat similarly, the refractory period following anaphylactic shock, and comparable sequelae in other types of immediate hypersensitivity, may well provide evidence bearing on the general subject of concern in this Session. We shall do no more here, however, than reflect that each of these subjects has its body of lore to be considered in any complete exploration of our subject.

Transplantation of Erythropoietic Tissue Into Irradiated Recipients. There is considerable current interest in the induction of persistent red cell chimerism in adult experimental animals, achieved through the injection of normal bone marrow into irradiated recipients. Sufficient dosages of x-radiation prevent an immediate antibody response against the foreign homologous tissue, so that bone marrow can become established as an active transplant. It continues to produce red cells of the donor type over considerable periods of time. The work of Lindsley, Odell and Tausche (48), based on the use of two closely related lines of rats distinguished by a pair of erythrocyte antigens and using rather high x-ray dosage (700 or 750 r), suggested an indefinitely long maintenance of the transplanted cells at a stable level achieved by about 35 days after the injection, and ranging from 10 to 95% of the circulating red cell population derived from the transplanted marrow. Main and Prehn (49) demonstrated that animals subjected to this treatment would accept skin transplants from the donor strain. It therefore appears possible to achieve successful homotransplants of skin in adult experimental animals through a two-step operation: first, irradiating the recipients and injecting bone marrow and second, establishing a skin transplant from the same donor or strain. Later work has revealed variations in the eventual stability of bone marrow transplants into irradiated recipients. A paper to be presented by Odell and Caldwell (50) at the current Federation meeting discusses results of one of these recent studies. It appears that the degree of relationship between donor and host and the amount of radiation to which the recipient is exposed are critical variables. With lower levels of radiation, and with diverse donor and recipient combinations, the injection of bone marrow may actually result in an *in vivo* reaction that may cause the death of the recipient (cf. 51). It should be emphasized that these brief remarks do not attempt anything like a complete review of this rapidly developing subject. A number of the most important published contributions have been omitted from this consideration, and other recent developments presented at the current Federation meetings or at the Gatlinburg Colloquium early in April were not available at the time of preparation of this manuscript.

Dixon and Weigle (52) have pointed out a clear difference between neonatal recipients and adults whose antibody-forming capacities have

been suppressed by x-rays. Potentially antibody-forming cells, injected into an irradiated rabbit, can make serum antibody in their new environment. Similar cells injected into neonatal rabbits, on the other hand, are unable to do so. Dixon and Weigle suggested that the environment provided by the neonatal animal may be deficient in a factor essential to an early phase of the process of antibody formation, while the body of the irradiated adult is not similarly deficient.

Billingham and Brent (53) have reported that when strain A mice were made tolerant to later transplants of CBA skin by the injection of adult CBA spleen cells into new-born A mice, the recipient mice in a number of instances showed marked anatomical deficiencies in their lymphoid tissues; many of the lymph nodes appeared to be completely absent. The donor spleen cells evidently undertook a 'graft versus host' reaction, resulting in the destruction of lymphatic tissues in the developing recipient. Similar reactions have been indicated in other work, and in the present connection they suggest that once competent cells have become established in their newborn host, the host can at least go on to elaborate any necessary factors involved in an immune response on the part of these competent cells. That these cells do in fact colonize the host lymphatic tissues, in the case of spleen cells injected into radiated recipients, was clearly demonstrated by Mitchison (54).

Immunological Paralysis and Related Phenomena. Felton (55) has shown that, while small doses of pneumococcal polysaccharides will immunize mice against living pneumococci, larger dosages (e.g. 0.5 mg) not only fail to immunize but actually prevent a protective antibody response against later exposure to the pneumococci. The long persistence of these polysaccharides in the tissues has suggested that the mechanism of this effect may be simply to absorb out antibody as fast as it may be produced. It seems possible that a 'steady state' phenomenon may be involved, such that an intracellular equilibrium is established 'trapping' antibody at the site of its production, by discouraging its dissociation as long as antigen remains in excess. Recently, Stark (56) has presented data indicating that such depots of antigen actually decline in their antigenicity as judged by their effects when inoculated into normal mice. The antigen in the tissue might be blocked by combination with antibody or antibody-like material, and thus lose its effective antigenicity. Such mechanisms, which have been described by Medawar (33) as peripheral, and applied to systems of immunological paralysis and enhancement but not to true tolerance, may involve afferent blocks, which prevent access of potentially antibody-forming cells to the antigen, or efferent, preventing the release of formed antibody into the circulation, or a combination or succession of such effects. Similar considerations may be applied to the 'enhancement' phenomena to be discussed in the next section.

One is tempted to analogize this situation with the effects of large doses of protein antigens injected into embryos and very young animals. At least in adults, however, no very persistent paralysis of an antibody response to protein appears to be achievable. Particularly provocative in this connection are the 'inactive' and 'refractory' phases sometimes encountered during continued immunization, observed, for example, by Taliaferro and Taliaferro (57) in rabbit responses to sheep cells. More recent study of this system by Talmage, Freter and Taliaferro (58) demonstrated a sharp decline of hemolytic activity during the fourth week of repeated injections, and a coincident rise in isophile combining capacity. The authors suggest that the changing hemolytic efficiency results from changes in the relative concentration of two antibodies, which reach their maxima at different times. There is an evident parallelism between this concept and the discussion earlier in the present paper of effects of injecting human red cells into newly-hatched chicks.

Enhancement of Tumor Growth. Snell, Kaliss and their co-workers have defined a system in which injection into a mature mouse of considerable quantities of lyophilized normal or tumor tissue may suppress a protective response on the part of the recipient, such that a transplanted tumor, which the untreated host would have caused to regress, will grow and produce the death of the treated recipient (59, 60). This 'enhancement' of tumor growth has some characteristics at least superficially in common both with immunological paralysis and with actively acquired tolerance to tissue transplants. A remarkable aspect of this system is that suppression of a protective immune response can be achieved by the injection of antisera to the normal or tumor tissue. In fact, recent data suggest that it may be the development of an antibody response that is the essential characteristic of the enhancement phenomenon. Other systems

have also indicated a depressive effect of circulating antibody on immune respnoses (61–63).

It appears that the tumor transplantation system is balanced on a knife edge, and that any of the variety of treatments that may cause delay or even mild suppression of a protective response on the part of the host may permit the transplanted tumor to obtain a foothold and to grow progressively, to produce the death of the recipient. Again, whether there are elements of the mechanism of enhancement that relate to acquired immunological tolerance in its restricted sense is a question that remains for further experimental work to settle.

DISCUSSION

The essential phenomena of immunological tolerance are defined in terms of the establishment and persistence of tissue transplants, achieved by the introduction of living homologous tissue cells into the embryo or neonatal individual. Perhaps it is well, at this stage of knowledge of this core problem of tolerance, to re-emphasize three aspects of this system that suggest caution in identifying it with other suppressions of immune responses: 1) the tolerance-inducing stimulus is a population of living cells; 2) the recipient is immature; and 3) the system is that of transplantation immunity, which is believed to be related, not to circulating serum antibodies, but to cell-borne and cell-mediated immune reactions.

With regard to point 1 above, we must repeat that, although extracted (apparently nuclear) antigens seem to be responsible for transplantation immunity in the adult, it has not so far been possible to induce transplant tolerance with these preparations. Furthermore, the types of cell preparations that have been used to induce tolerance include, or at least conceivably may include, cells capable of giving an active response against host cells or tissue components. Commonly, spleen cells, lymphatic cells, or heterogeneous cellular preparations that include components of the reticulo-endothelial systems have been used to induce transplantation tolerance. Even in the case of tolerance induced by injections of whole blood, it appears that the faculty of producing tolerance is restricted to the leucocyte fraction. In the naturally tolerant bovine twins, and in the tolerance induced by egg-to-egg parabiosis, as well as the experimental induction of tolerance by the injection of tissue cells, there is as yet no compelling reason to rule out the possibility that tolerance in the restricted sense depends upon the establishment and persistence of donor cells. In this connection, the observation by Billingham, Brent and Medawar (14) of the disappearance of skin graft tolerance coincident with the regression of erythrocyte mosaicism to the point of loss of the donor cell type from the circulation of a parabiotic chick suggests a close relationship between the two phenomena. It may also be important to note that this disappearance of red cell chimerism and the concurrent development of intolerance toward an established skin graft occurred in chicks that still continued to display a considerable degree of inhibition of the development of agglutinating antibody upon immunization. Hasek's observation, mentioned earlier, of suppressed precipitin formation in a hen that had lost its initial chimerism with turkey cells is a similar case in point (13).

The specificity of the graft-tolerance system demonstrates that it cannot depend on a generalized lesion in the cellular defense mechanisms of the host, achieved through wholesale graft-versus-host reaction on the part of competent donor cells. A host rendered tolerant to donor tissue of one strain remains capable of reacting normally against tissues of another. That the effect is a central one on the host defenses, and not dependent on adaptations on the part of the graft, is most elegantly demonstrated by the experiments in adoptive immunity, in which tolerated skin transplants have been shown to regress after the injection into the host of competent spleen cells from normal individuals of the host inbred line. This experimental evidence demonstrates that the graft is continuing to elaborate its own specific antigens, which are capable of evoking an immune response in normal cells, and that the graft remains vulnerable to the effects of such responses (cf. 14). Changes in the transplanted tissues may also occur, either as a function of the selection of antigenic variants or possibly as a result of 'type transformation' or 'serotype transformation'; and Cannon, Terasaki and Longmire (64) have properly emphasized that there may well be systems in which graft adaptation plays an important part. These authors cite evidence that tolerated skin grafts to young chicks may be a case in point. Woodruff and Simpson (65) have presented somewhat similar arguments for rat skin grafts, and Kaliss (60) considers that tumor changes, induced or selected by host antibody responses,

may be important in the enhancement phenomenon. Related matters will be discussed by Dr. Koprowski. The maintenance of their original antigenic type of erythrocytes derived from exchanges of erythropoietic tissue in embryonic cattle, sheep, rats, chickens and man, however, and the phenomena of adoptive immunity as well as other evidence marshalled by Medawar and his group (cf. 14), demonstrate convincingly that adaptive changes in the transplanted tissue are not a necessary condition for tolerance.

It is difficult at present to assess the significance of the fact that the transplantation tolerance system appears to have no necessary cause-and-effect relationship with the elaboration of serum antibody. The relationships between the cellular and the soluble immune components are obscure at present; perhaps we should be content here with a cautious recognition that treatments that interfere with one of these channels of immune response need not affect the other. The mechanisms by which the injection of a soluble antigen or of non-surviving cells into an embryo or an immature individual results in a persistent suppression of the circulating antibody response may be quite different from those that induce transplant tolerance in the cellular system. When we turn to procedures that subdue or inhibit antibody responses in adults we need to be especially cautious in assuming that we are dealing with the core problem of immunological tolerance, in its restricted sense, at all.

Even within the category of circulating antibody responses, we need to recognize the complexity of the phenomena with which we are dealing. The tissue-sensitizing, circulating antibody-like reagins, the incomplete antibodies of various types, and the complete antibodies of saline agglutination and precipitation have, as yet, little-understood relationships to each other in terms of the total immune mechanism. Divergence of one of these channels of response into another, blockage of one while another is not blocked or may even be stimulated, and interactions among these systems need to be distinguished from tolerance effects, at least until it can be demonstrated that one or more of these is causally involved in such effects. And there are components of the immune machinery, specific and aspecific, not even mentioned here; no doubt there are others still to be discovered and defined.

Various aspects of tolerance have been subjected to different kinds and degrees of explanation (e.g. 66–69, and references cited in particular connections throughout this paper). It is tempting to attempt a general explanation here or at least a scheme that might relate the various aspects of suppression of immune response to each other. Such a scheme would need to be built on a logical and consistent and reasonably complete scaffolding, representing not only the mature machinery of immunity in all its parts, but its origins and its ordinary changes during development as well. This is a challenge I am not prepared to meet at this time.

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IMMUNOLOGICAL TOLERANCE IN TUMOR TRANSPLANTS

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IF A DISASTER befell you in which you suffered from burns affecting such an extensive surface area of your body that a protective cover for the exposed part had to be found, one of the surgical procedures proposed would be the transplantation of skin from some charitable individual. This is commonly referred to as *skin grafting*. It is, unfortunately, a temporary expedient. Only under three circumstances would the skin graft survive and be accepted; under all other conditions the graft, after a period of semi-survival, would be rejected and would wilt away. Although this has been a subject of controversy for decades, it remains an indisputable fact. Credit should be given to a German surgeon, Erich Lexer, who in 1910 published his investigations on a large series of patients on whom, without exception, skin grafts from other individuals were unsuccessful (1).

The three unusual circumstances which would favor retention of the skin graft and its permanent survival are: *1*) transplantation of skin from another part of your own body, referred to as 'autografting,'; *2*) transplantation of skin from your identical twin, if you are lucky enough to have one; and *3*) if you, as a recipient, suffer from a condition called either 'agammaglobulinemia' or —perhaps better—'dysgammaglobulinemia', a condition in which the level of the gamma globulin fraction of your serum is almost nil (2). This peculiar ailment, which I do not wish on you any more than the other gloomy situations mentioned before, may enable you to accept skin transplants from nonrelated persons, with a good chance of survival of the grafts.

This predicament of rejecting skin grafts—if it is a predicament, and I shall return to that later—we share with all other species of animals in which skin transplantation has been tried, the single exception being the hamster. Skin transplants can be made from Syrian and Chinese hamsters (3) to genetically unrelated recipients of the same species; the graft will be accepted in a majority of cases and will survive for a long time. What happens to a skin homograft (and by that term we understand transplantation of tissue between two animals of the same species but genetically unrelated) is shown on slides (figs. 1–7) which Drs. Medawar and Brent (4) have kindly lent to me. On figure 1 you will see the appearance of a skin homograft obtained originally from an adult white mouse and transplanted to an adult brown mouse. The genetic line of the donor white mouse is known as A strain, and that of the recipient as CBA strain. This is the 11th day after transplantation, and the rejection of the graft is complete as indicated by the disintegration of the graft, which is drying to form a scab.

Figure 2 shows a 2-week-old White Leghorn chicken 8 days after it was grafted with the skin of a Rhode Island Red. The homograft has been rejected, has broken down and formed a scab. Figure 3 illustrates the chest surface of a rabbit 12 days after it received autografts and homografts. Its own skin grafts have been accepted; the homografts have broken down and been rejected.

Is the rejection of tissue grafts, since the fate of skin transplants is shared by any other tissue or organ of the body, a predicament for these animals and for ourselves, or is it a good fortune? Would we be luckier if we could accept grafts from any donor, and thus have new kidneys, new skin or new testicles when needed? The chances are that an animal species in such a state of impaired biological activity would probably be destroyed by a number of foreign invaders, such as bacteria, viruses etc., against which it would be defenseless, long before any grafts could be of any avail or successful. I would like to dwell longer upon this entire concept, referring to Medawar's brilliant exposition (5).

It involves essentially the relationship between a pregnant mother and the embryo or fetus. The fetus *in utero* is, of course, a highly biologically active specimen: it grows, it excretes, and—as all of you who are mothers know—it kicks. The effect of this biologically active tissue on the

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organism of the mother may be profound, and she may respond to the stimulus by becoming immunized. This problem has been studied extensively for many years, and has already been discussed by Dr. Owen. I would like to mention that certain congenital abnormalities may be explained by the fact that the mother's organism became immunized against the tissue of the fetus. But what about the reverse? The mother's organism is also biologically active, and it may provide stimuli to which the fetus reacts. Can the latter become immunized? Apparently not—and happily not. Should the fetus become immune to the body constituents of its mother, the fetal antibodies might react against some or all constituents of its own body and lead to self-destruction.

Luckily, instead of being immunized, the fetus becomes tolerant. Again, the concept of tolerance has been described and defined by Dr. Owen, and need not be repeated here. The fetus cannot react immunologically against its own tissues unless some foreign artificial intervention takes place, and this state persists during the lifetime of an individual. This, of course, is not the case for tissues other than his own. The exceptions, as mentioned before, are cases where there is a common blood circulation, dizygotic cattle twins

FIG. 1. Appearance of skin homograft from an adult white mouse and transplanted to an adult brown mouse. (From *Philos. Tr. Roy. Soc. London, s.B.* 239: 357, 1956.)

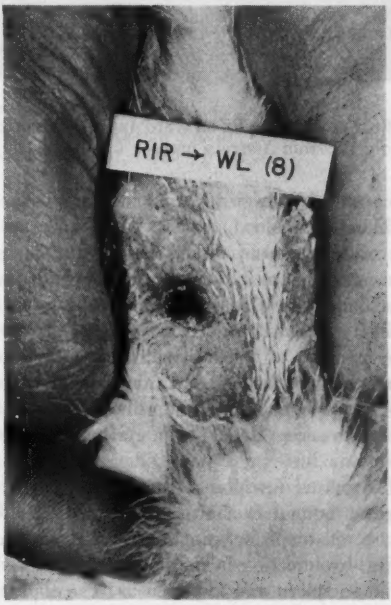

FIG. 2. Two-week-old White Leghorn chick 8 days after it was grafted with skin of a Rhode Island Red. (From *Philos. Tr. Roy. Soc. London, s.B.* 239: 357, 1956.)

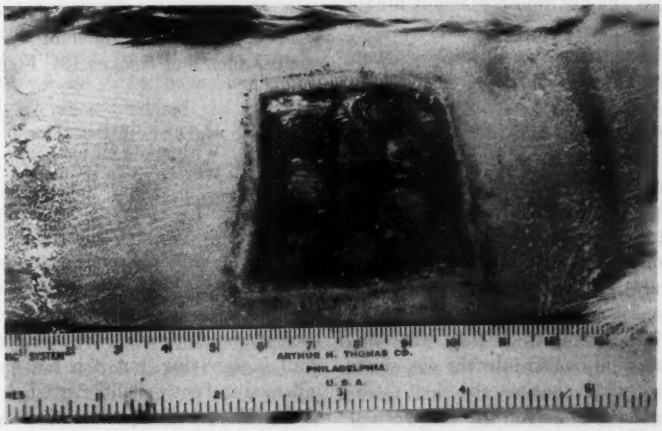

FIG. 3. Chest surface of a rabbit 12 days after receiving autografts and homografts.

FIG. 4. CBA mouse on 173rd day after receiving skin graft from A-strain mouse.

discovered by Owen (6), and identical twins in mankind. The blood elements educe tolerance in the other organism which enables it to accept tissue grafts from its twin during post-embryonic life. This natural tolerance can be imitated, as Billingham, Brent and Medawar (7) have shown. The injection of whole blood, blood-forming organs, kidney cells etc. from animals of one strain into fetuses of another strain will render the latter willing to accept doner-strain grafts during post-embryonic life.

The actual acceptance of foreign skin by a tolerant animal is shown in figure 4. A CBA mouse was made 'tolerant' to the A-strain white mouse by injection in its embryonic stage with A-mouse spleen and kidney cells. The slide was made on the 173rd day after the CBA mouse had received a skin graft from an A-strain mouse. Compare this perfectly healed and accepted transplant with the scab shown previously, when an 'unpretreated' mouse rejected the A-mouse graft.

The individual specificity to tolerance is demonstrated in figure 5. Here, a CBA brown mouse, tolerant to strain A homografts, has rejected a skin graft from a strain genetically unrelated to the A strain.

Tolerance in birds has been extensively studied. Figure 6 shows a White Leghorn chick 3 months after receiving a skin transplant from a Rhode Island Red. Compare the luxuriant red plumage with the miserable scab shown before (fig. 2). In this experiment, the donor and the recipient were made into artificial twins when they were embryos. Windows were drilled into the egg shells on the 10th day of incubation, the shell membranes were removed to expose the vascularized membranes (chorioallantois) which were then

FIG. 5. Rejection of skin graft from strain genetically unrelated to A strain by CBA brown mouse, tolerant to A strain. (From *Philos. Tr. Roy. Soc. London, s.B.* 239: 357, 1956.)

aligned and the eggs were joined by insertion of a vascular bridge. Thus, the embryos were provided with a common blood circulation until the time of hatching, and became tolerant to each other.

Attempts to transplant tissue between two different species end, with rare exceptions, in failure. This is called 'heterotransplantation.' The artificial twinning, which is called parabiosis, has been done between different species—for instance, duck and chicken (8). Figure 7 shows a duck:

> D was a duck
> With spots on his back
> Who lived in the water
> And always said 'quack.'
>
> (Lear's *Book of Nonsense*)

which has been in parabiotic union with a chicken as an embryo. The chicken skin heterograft was made shortly after the duck hatched, and has been under observation for 26 days. The heterograft is surviving, but it is not happy: smaller, inflamed and not well differentiated. Heterologous skin grafts are always imperfect because of the wide genetic gap between different species.

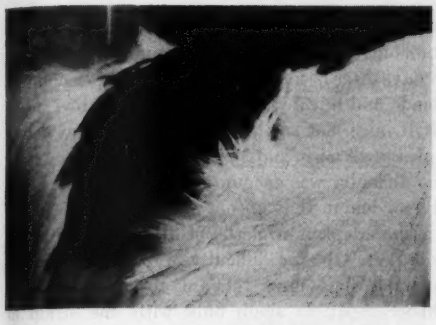

FIG. 6. White Leghorn chick 3 months after receiving skin transplant from a Rhode Island Red.

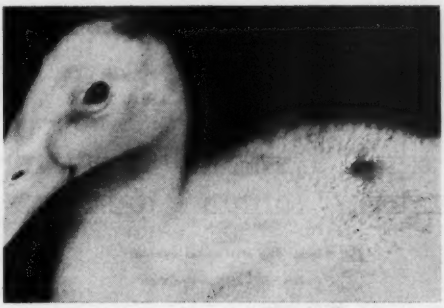

FIG. 7. Duck in parabiotic union with a chicken as an embryo. (From *Philos. Tr. Roy. Soc. London, s.B.* 239: 357, 1956.)

The state of immunological inactivity when the fetus may become tolerant to biologically active stimuli has been called by Hasek the 'adaptive period.' Although, in general, this state of affairs is much more beneficial for the fetus than if it were able to produce antibodies upon antigenic stimulation, sometimes the fetus may be made more vulnerable. For instance, malignant melanomas (pigment-bearing tumors) are known to be transferrable from mother to fetus (9). The latter, inactive and defenseless, perishes after the invasion of its organs by the neoplasm, since no immunity reaction is provoked by the tumor tissue.

The biological laws governing the transplantation of tumors have been recognized for some time. In 1903, Jensen (10) had already observed the difficulties in grafting tumor tissue on mice of different 'races.' It has subsequently become quite apparent that the immunological barriers preventing transplantation of malignant tissue from one host to another are similar in nature to those which play such a prominent role in the transplantation of normal tissue between genetically incompatible hosts. Since the mechanism of immunological tolerance rendered the barriers ineffectual to normal tissue transplants, the same method was applied to neoplastic or malignant tissue.

The initial attack was to find a tumor which originated in a genetically defined strain of mice and which remained compatible with this strain, i.e. grew progressively, killing the host, but which was unable to do so when transplanted into any other strain of mice. Thus the choice fell on a tumor which is called, to the eternal confusion of the chemists, the 6C3HED tumor. The chemical incompatibility between C(arbon) and H(ydrogen) refers to an inbred strain of mice in which this particular tumor was originally induced by the application of a chemical substance. The original tumor has undergone numerous transplants in the C3H strain of mice and has remained faithfully compatible with the host. By 'compatibility' we do not mean conjugal bliss, but rather a definition of marriage which runs something as follows: "marriage is an attempt by two individuals to undertake the seemingly impossible task of becoming one. This is achieved when the woman becomes everything and the man becomes nothing." If you substitute 'tumor' for female and 'C3H mouse' for male, you will see what is meant by 'compatibility'; the host becomes nothing, the tumor becomes everything. Having secured a non-transplantable tumor, it was then important to choose a strain of mice which would normally reject the 6C3HED tumor. The choice fell on ICR mice. This is a Swiss strain, not inbred, which is characterized by a large number of progenies. The disadvantage of using a non-inbred strain was outweighed by the greater resistance of these mice to laparotomy and to fetal inoculation. An attempt was thne made to impair the mechanism of immunity of ICR mice to 6C3HED tumor by making them tolerant.

In the first two experiments, on the 17–18th day of pregnancy, ICR females were operated on through the abdominal wall. The fetuses enclosed *in utero* were injected with 0.01 ml of whole heparinized blood obtained either from a C3H mouse or from the F_1 (first generation) of a cross between C3H and DBA mice. The mortality risk for the mother mouse and her embryos, following this rather drastic procedure, is surprisingly low and the birth rate is high.

As has been shown on numerous occasions by

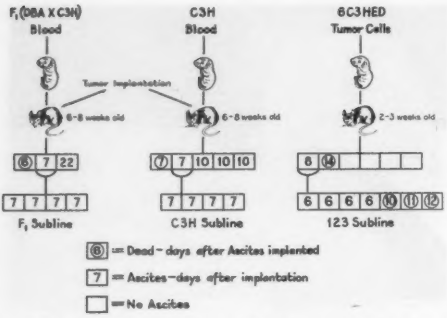

FIG. 8. Induction of actively acquired tolerance in Swiss mice to 6C3HED ascites tumor. (Reproduced from *Proc. Roy. Soc., London, s.B.* 146: 37, 1596.)

Billingham *et al.* (4, 7), newborn mice if injected as embryos with blood derived from C3H mice should become tolerant to a skin graft originating from a C3H mouse. What about susceptibility to a C3H tumor? As shown in figure 8, 6–8 weeks after birth these supposedly tolerant mice received implants of the 6C3HED tumor into their abdominal cavities. All developed ascites. The abdominal walls were tapped and ascitic fluid withdrawn. Numerous tumor cells were present in the fluid, and it was clear that the original implant had succeeded in establishing the tumor in the hitherto insusceptible host. One of 3 mice in the first experiment, and one of 5 in the second experiment died of ascites. Further transplantation of the tumor cells intraperitoneally into normal, not pretreated, adult ICR mice established this tumor line beyond any doubt in ICR mice. After subsequent passages, the tumor cells grew progressively, causing death from malignancy of all the inoculated animals (11).

In the third experiment of the same series, instead of injecting ICR fetuses with C3H mouse blood, actual 6C3HED tumor cells were implanted. This direct method of injecting the tissue is possible in the case of tumor cells. Skin transplants cannot be made on embryos *in utero* and therefore tolerance has to be induced by the indirect method of injecting other substances. In order to be able to determine more precisely the actual number of embryos injected with tumor cells, the inoculum was colored with a vital dye, such as trypan blue. Thus, in the first experiment, 6 blue and 2 pink babies were born. Two of the 6 blue babies developed subcutaneous tumors in the neck region 2–3 weeks after birth. The histologic pattern of the tumors was identical with the original 6C3HED tumor grown subcutaneously in C3H mice. One tumor was excised 8 days after becoming palpable. A suspension was made and injected intraperitoneally into 7 adult ICR mice, not treated previously. Ascites developed in all mice, and 3 died of malignancy. Again, no difficulty was encountered in establishing this tumor line in ICR mice.

The adaptation of the 6C3HED tumor is the simplest. Greater difficulty was encountered in making ICR mice tolerant to another tumor histocompatible again only with one strain of mice, the brown DBA strain. The tumor is called the DBA/2 lymphoma. Attempts to transplant it into ICR mice are shown in figure 9.

Following intra-uterine inoculation of tumor cells into 3 pregnant ICR mice, 5 out of a total of 17 blue babies developed neck tumors 10 days after birth. When suspensions of these tumors were injected into adult ICR mice, ascites formed 8 days after inoculation. Serial passage of the ascites fluid in ICR mice ended, however, in failure to establish this tumor line in ICR mice. Another stratagem was therefore employed. Neck tumors, which developed in the first generation of ICR mice injected in the embryonic stage, were removed, made into a suspension, and injected into ICR embryos, instead of in adult mice (fig. 9). Four out of 4 blue babies developed tumors 8 days after birth. Another attempt to propagate this tumor was made at this passage level and failed. A suspension of these tumors was once again injected into ICR embryos, and one out of 6 baby mice developed tumors. One more intra-uterine passage was made, and then the tumor suspension was injected into 6 adult ICR mice (fig. 9).

One of these developed ascites which was passaged to 3 other mice. Two developed ascites, and fluid containing a large number of tumor cells was implanted intraperitoneally into 8 ICR mice. All developed ascites, and 7 died of malignancy. Subsequent passages in adult ICR mice confirmed the successful establishment of a second lymphoma in ICR mice (12).

Another strain-specific mouse ascites tumor, the P-288, was adapted to ICR mice. This tumor grows ordinarily in the DBA/2 and DBA/1 mouse sublines. On figure 10 it may be observed that after two subcutaneous passages in newborn ICR mice, the tumor started to grow in ascites form in adult, non-pretreated ICR mice. Following 16 intraperitoneal passages, it became uniformly lethal to the new host.

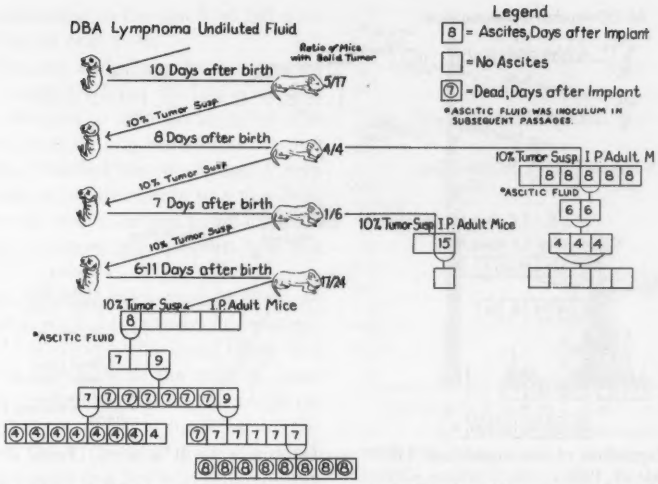

FIG. 9. Adaptation of DBA lymphoma ascites tumor to ICR mice. (From *Proc. Roy. Soc., London, s.B.* 146: 37, 1956.)

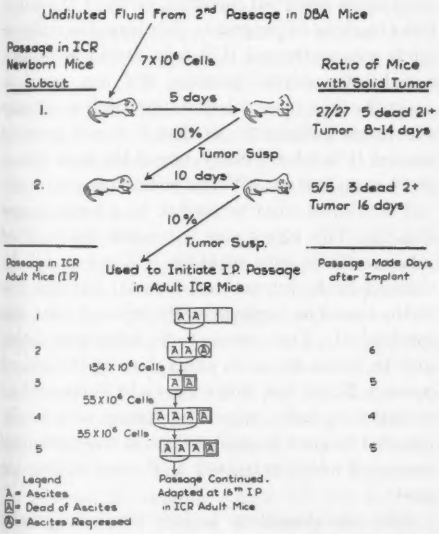
Undiluted Fluid From 2nd Passage in DBA Mice
Passage in ICR Newborn Mice Subcut
7X10⁶ Cells
Ratio of Mice with Solid Tumor
1.
5 days
27/27 5 dead 21+ Tumor 8-14 days
10 % Tumor Susp
2.
10 days
5/5 3 dead 2+ Tumor 16 days
10 % Tumor Susp.
Passage in ICR Adult Mice (I.P.)
Used to Initiate I.P. Passage in Adult ICR Mice
Passage Made Days after Implant
1.84 X 10⁶ Cells
55 X 10⁶ Cells
55 X 10⁶ Cells
Passage Continued. Adapted at 16th I.P. in ICR Adult Mice
Legend
A = Ascites
Ⓐ = Dead of Ascites
Ⓐ = Ascites Regressed

FIG. 10. Adaptation of P-288 DBA specific ascites tumor to ICR mice.

Greater ingenuity was required for subsequent homo-adaptations of tumors, as compared to the experience with the 6C3HED neoplasm. This confirmed the eternal bugbear of the biologist: that the first experiment is often too easy, and that following attempts will be more difficult. This foreboding proved to be justified when an attempt was made to break through the species barrier and adapt rat tumors to a mouse host. As mentioned before, this procedure is called 'heterotransplantation.' Parallel with our efforts in the field of mammalian tissue, attempts were being made in three other laboratories to induce tolerance in turkeys to chick tissue. The Rous Sarcoma is a chicken tumor of viral origin. Turkeys more than 3 weeks old will reject the tumor (13). Simonsen (13), in a preliminary experiment, injected turkey embryos on the 13th day of incubation with 0.1 ml of blood cells from young chickens. Following hatching, three 8-week-old turkeys were injected intramuscularly with a cell-free preparation of Rous Sarcoma grown in chicks. Two of 3 turkeys developed tumors at the site of inoculation, while all of the control birds rejected the tumor. Svoboda and Hasek (14) injected blood obtained from adult chickens into either turkey embryos intravenously on the 15th day of incubation, or into newly-hatched turkeys. Rous tumor was implanted intramuscularly into 23 birds at an age when all control birds were found to be refractory. With two exceptions, these turkeys developed tumors and died from the malignancy.

Harris (15) has made a thorough investigation of tolerance in turkeys to the Rous Sarcoma agent. He injected newly-hatched turkey poults with 0.3 ml of whole citrated chicken blood intravenously, and produced tolerance to Rous tumor. The period during which young turkeys can be made tolerant by the inoculation of chicken blood was found to be very short, not exceeding 72

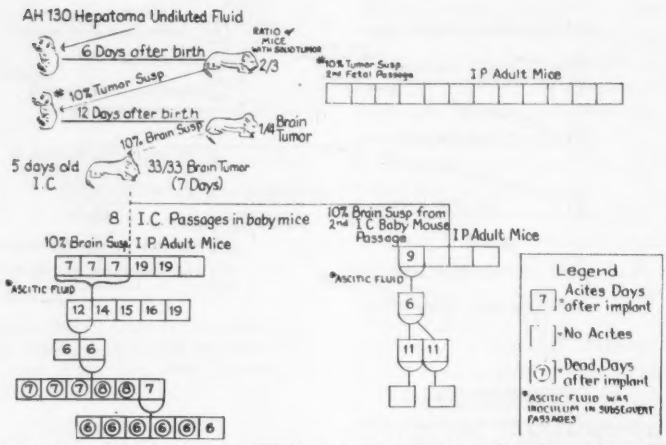
AH 130 Hepatoma Undiluted Fluid
6 Days after birth
RATIO OF MICE WITH SOLID TUMOR
2/3
10% Tumor Susp 2nd Fetal Passage
I P Adult Mice
10% Tumor Susp
12 Days after birth
1/4 Brain Tumor
10% Brain Susp
5 days old I.C.
33/33 Brain Tumor (7 Days)
8 I.C. Passages in baby mice
10% Brain Susp from 2nd I.C. Baby Mouse Passage
I P Adult Mice
10% Brain Susp
I P Adult Mice
*ASCITIC FLUID
*ASCITIC FLUID
Legend
Acites Days after implant
No Acites
Dead, Days after implant
*ASCITIC FLUID WAS INOCULUM IN SUBSEQUENT PASSAGES

FIG. 11. Adaptation of rat hepatoma AH130 ascites tumor to ICR mice. (From *Proc. Roy. Soc., London, s.B.* 146: 37, 1956.)

hours after hatching. Serum or plasma of chickens did not induce tolerance; the factors influencing the development of tolerance were found to be in the formed element of the blood. Judging from what we now know about the nature of 'natural' antibodies occurring in the sera of older chickens refractory to the Rous Sarcoma (16), it is possible that by injecting turkey embryos or newly-hatched poults production of a similar type of antibodies in turkeys was prevented. Such antibodies (in the case of older chickens) are not directed against the virus of Rous Sarcoma, but against the malignant cell itself (16).

Attempts at heterotransplantation of mammalian tumors by the induction of tolerance have been very few. Bollag (17) observed that rats injected as embryos with normal mouse cells were more susceptible to implantation of Crocker mouse sarcoma. Conversely, Grozdanovic (18) observed incomplete tolerance to Crocker mouse tumors in rats injected as embryos with mouse tissue. Although the tumor sometimes grew slightly larger in such an animal it invariably regressed 10 days after implantation.

That heterotransplantation of tumors through the breaking of the species barrier presented a formidable task became evident when an attempt was made in our laboratory to adapt a rat tumor to mice. This tumor originally developed as a liver tumor in a rat, but has subsequently been grown in ascites form intraabdominally. It is known as the AH130 tumor. Utilizing the procedures used to educe tolerance in the successful homotransplantation of mouse tumors, AH130 cells were injected into ICR fetuses. Neck tumors were again observed following birth of the mice, but attempts to propagate this tumor serially in adult non-pretreated ICR mice ended in failure. Serial intra-uterine passages did not seem to make the mice more tolerant, nor did they change the tumor sufficiently so that it would grow in normal ICR mice. Finally, one of the mice inoculated as an embryo with the second passage material developed what seemed to be a brain tumor (fig. 11). This tumor was harvested and injected intracerebrally into suckling ICR mice. All developed brain tumors and a serial passage line of the tumor in intracerebrally injected mice was established. The attempt to establish lethal growth of the tumor in adult mice at the second passage failed but following eight intracerebral passages in baby mice, the tumor was finally adapted to grow in ascites form in the peritoneal cavity of adult untreated ICR mice killing the host.

This encouraged us to face the even greater task of attempting to grow another rat tumor, the Yoshida ascites, in mice. The history of this adaptation (19) will not be related as it, essentially, would be a repetition of the procedures described above. It will suffice to say that we succeeded in growing the tumor serially in ICR mice after 42 passages in the brain tissue of suckling mice.

One important point should be emphasized: all of the tumors described above, after passage through an immature organism of a mouse strain originally resistant, changed so that they could

not only be maintained in the new host but were uniformly lethal to that host.

Is the mechanism underlying transplantation of tumors to resistant hosts the same as that operating in the case of skin homografts? The answer can be given both in the affirmative and in the negative. The first step is similar. A very young organism, either an embryo or a new-born animal, is injected with a foreign tissue to which it subsequently becomes tolerant. But here the parallel ends.

Foreign skin, growing on a tolerant host, retains all the properties of the donor. Re-examination of figure 4 illustrates this point. Here is a skin graft with hair tufts of the original color, although it is firmly attached to the recipient. The same striking evidence is furnished by the observation of Good (2) who grafted a patch of skin from a woman to a boy who suffered from agammaglobulinemia, a condition mentioned at the beginning of the lecture. This skin graft was accepted, but the chromatin retained original female characteristics, although it was firmly attached to an otherwise normal subject of the opposite sex. The sex of the tumor cells has not yet been determined, but a profound change is observed in the properties of a tumor once it has been transplanted successfully from a tolerant host to a normal mouse. These changes will be summarized very briefly.

The cell volume increases and the number of chromosomes per cell nucleus rises. The original tumor cell is characterized by a number approaching 42 (fig. 12); the transplanted tumor cell, depending on the tumor, usually has double this number. Accompanying this phenomenon is a rise in the DNA and RNA per cell, the values for the transplanted tumors being double or triple in relation to the original tumor. Finally, one more important change should be stressed. In general, the original tumor cell will not support growth of viruses artificially introduced. The transplanted tumor not only supports the growth of any one of a multitude of viruses but, by so doing, commits suicide, since all malignant cells are destroyed by the multiplying virus. Thus, the initial inoculation of the tumor cells into a young, non-reactive animal probably does something to the animal; conditions it to a certain extent to become tolerant to the tumor, but subsequently the tumor itself changes as it grows under the influence of its new environment. The striking difference between the characteristics of the transplanted and original tumors may lead

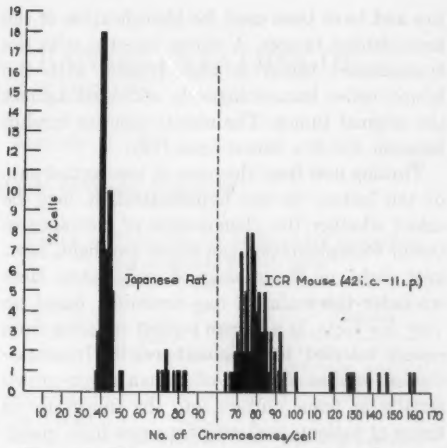

FIG. 12. Chromosome number histograms of Yoshida ascites lines.

the purist to believe that we are dealing with an emergence of new types of tumors in the resistant host induced by some factor present in the old transplants. This is neither the place nor the time to present all the arguments against this hypothesis. I will only mention the most important factors.

Rat tumors transplanted into mice through many successful passages retain their lethal character for rats. Although the number of cells per inoculum per rat has to be large to be lethal, no bona fide mouse tumor has displayed such a property. The other identification of the transplanted tumor can be accomplished by treatment of the animals with immunologically activated lymph nodes. Again, if we return to skin transplantation, you will recall the beautiful tufts of original A hair on a recipient CBA mouse (fig. 4). This graft can be destroyed in the following way: skin obtained from an A-line donor is transplanted into a normal CBA mouse. Within 8–11 days, it will wilt. If axillary lymph nodes are removed, and these lymph nodes are the ones which receive 'drainage' from the wilting skin graft, then a sort of 'antibody' is found in their cellular extracts. This antibody makes itself manifest if injected into a tolerant CBA mouse bearing an accepted A-line skin graft. Injection of the lymph nodes makes the mouse intolerant (to use this excellent term coined by Szilard—personal communication), and the beautiful tuft of white A hair is destroyed (4). Such lymph nodes obtained from mice injected with tumor grafts, instead of skin, have anti-tumor proper-

ties and have been used for identification of the transplanted tumors. A mouse injected with the transplanted tumor is then treated with the lymph nodes immunologically activated against the original tumor. The results indicate kinship between the two tumor lines (12).

Turning now from the more or less factual part of the lecture to the hypothetical, it may be asked whether the phenomenon of tolerance in tumor transplantation can throw any light, however slight, on the etiology of malignancy. Here we enter the realm of day-dreaming, based on very few facts. Is a human subject suffering from cancer 'tolerant' to malignant growth? Implantation of cellular material of human origin grown serially in tissue culture into the subcutaneous tissue of patients, suffering for years from malignancy, leads to the formation of tumors. The same tissue culture cells implanted into normal, healthy adults are rejected after a violent display of defense reaction (20). Are the cancer patients so debilitated that their defense mechanism is inactive, or are they 'tolerant' to implantation of tumors? Another intriguing problem in the same general category is the observation of several workers (21, 22) on tumor-inducing filtrates. Suspensions of subcellular elements obtained from several mouse leukemias can induce cancer in mice if injected into the animals within several hours after birth. There is only one exception to this age limit (23). Is tolerance operating? Are the subcellular particles making the injected host tolerant in such a way that, months later, when confronted with a malignant growth its defense system cannot recognize the tumor as foreign tissue but considers it as its own and does not muster an army of antibodies for counter-attack? These are a few of the pipe-dreams which may eventually form a link, even though not too strong, between tolerance, transplantation immunity and cancer.

Several months ago, I was invited to give a more specific talk on this subject at a meeting attended by specialists in this field. On finishing my lecture I realized, to my horror, that I was addressing a glassy-eyed audience, few of whom were interested in or understood what I was describing. Fearing the same reaction from tonight's audience, I have sought in advance the advice of Dr. Szilard, one of the outstanding scientists of this age. "How should I approach 'lecture-wise' an audience consisting of people from such diverse vocations as nutrition, biochemistry, physiology, pharmacology etc.?" His advice was to treat you as completely ignorant of the subject matter but possessing infinite intelligence. I hope I have succeeded in changing the first classification, but I am certain that in doing so I have exposed my own ignorance—perhaps more specialized, but ignorance nevertheless—about a subject which remains one of the most interesting problems in the field of biology.

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SUMMARY OF STUDIES OF TRANSPLANTATION OF HOMOLOGOUS TISSUES

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IN OUR STUDIES of tissue transplantation we have made microscopic observations of tissues and cells in living mice, using both the transparent chamber (1, 2) and diffusion chamber methods (3). The transparent chambers have been modified depending on the detail desired.

Diffusion chambers have been developed in which implanted cells survive and proliferate for long periods of time, although separated from the tissues of the host by membranes of varying porosities. These chambers may be placed either intraperitoneally or subcutaneously. They can be removed for histological processing and microscopic study after varying intervals of time. The membranes have been used also in the construction of transparent chambers (3, 4).

These techniques have been used to study problems of survival of homografts in mice. It is generally accepted that the reaction of the host to homografts is based on a mechanism of actively acquired immunity. Whether destruction is mediated by cells, or humoral antibodies, or by both, and the possible role of vascular reactions were among the problems studied.

Inbred strains of mice which provide a genetically uniform population have been used. Grafts within the same inbred strain are referred to as isologous. These usually survive, as do grafts between identical twins. Grafts between mice of different inbred strains will be referred to as homologous. These are usually destroyed, as are grafts between human beings, unless they are identical twins.

In studies utilizing the transparent chamber and diffusion chamber methods, two experimental situations have been found in which homografts have survived indefinitely in an incompatible host.

Transplants of small fragments of tissue (less than 1 mm diameter) survive even if they do not become vascularized (5). Indeed, such grafts may be deliberately placed so that a blood supply is not established. Not only isografts, but also homografts survived indefinitely as unvascularized grafts in the subcutaneous site in mice. The homografts survived because they did not initiate an immunologous reaction in the host whereas vascularized grafts always initiated immunity. However, the unvascularized homografts were destroyed if the mouse was immunized by a vascularized graft elsewhere in the same host.

TABLE 1. SUMMARY OF STUDIES ON SURVIVAL OF HOMOGRAFTS IN MICE

	Graft Vascularized	Graft Not Vascularized	Graft in Diffusion Chamber	
			Cell-penetrable chamber	Cell-impenetrable chamber
Fate of Graft				
Isologous host	survival	survival	survival	survival
Homologous host	destruction	survival	not determined	survival
Immunized homologous host	destruction	destruction	destruction	survival
Induction of immunity in host by homologous graft	immunity induced	immunity not induced	not determined	immunity not induced

The second method by which the destruction of homografts was prevented was by placing the tissue in diffusion chambers. As in the case of non-vascularized homografts, homologous tissues in cell-impenetrable diffusion chambers survived (3, 6) and did not initiate immunity (6). In contrast to the destruction of non-vascularized homografts in immunized hosts, homografts in cell-impenetrable diffusion chambers survived, indicating that the membranes prevented the destruction of the homograft.

Homografts were destroyed only in chambers made of membranes which allowed the passage of cells of the immune host (7). Evidence was obtained that the membranes did not block

the passage of humoral antibodies (7, 8). It was concluded, under these experimental conditions, that cytotoxic activity against these homografts was associated with cells of the immune host.

Apart from questions of the mechanism of homograft destruction, it is, we believe, of considerable significance that under these two special conditions (unvascularized or within a diffusion chamber) homografts have survived for many months in incompatible hosts.

This work has been carried out in cooperation with Doctors R. M. Merwin, R. T. Prehn, and J. M. Weaver, National Cancer Institute, Bethesda, Md. For skilled technical assistance our thanks are due to Mr. Roy Moore.

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AMERICAN PHYSIOLOGICAL SOCIETY

Symposium on Bioclimatology[1]

Chairman: DOUGLAS H. K. LEE

A PROSPECTUS OF BIOCLIMATOLOGY: HISTORICAL OBSERVATIONS ON ITS NATIONAL AND INTERNATIONAL GROWTH

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DISCOMFORT, pain, disease and death are natural events which jeopardize man's existence. On the one hand, they may strike suddenly. On the other, ill health may become chronic and exhibit exacerbations, relapses and remissions. Man sought to explain these phenomena. Out of a groping folklore arose medicine and the medical sciences. Early medicine recognized the oneness of man with his environment and the environmental viscissitudes were seen as the cause of pain and sickness. The concept of environment did not restrict itself to the earth's atmosphere (wherein the changes are called weather) but extended it into the cosmos and envisaged that the sun, moon and stars ruled over man's destiny. Hippocrates had a more conservative view of environment. He was not influenced by this astrology (1). He taught that one should learn meteorology before studying medicine (2). Hippocrates was perhaps the first bioclimatologist, for he investigated the effects of weather and climate on man and laid down the first set of working bioclimatological principles (2).

In the succeeding two thousand years the Hippocratic concept of man-environment (*physis*) (3) suffered a waning acceptance by medical scientists. For a time, even astrological medicine was more popular (4). More recently bacteriology became the dominant hypothesis. The patient receded into the background and the Cnidian theory (5) that disease was the object of medical study prevailed. Although one cannot gainsay the harvest of knowledge reaped, it is becoming increasingly evident that such an approach to the problems of human health and efficiency has not been sufficiently holistic. The pendulum has begun to swing back to the doctrine of the Coan School (5). The patient has been rediscovered and, with that discovery, a new idea of man-environment is emerging. The point of view is ecological and has been called human ecology (6, 7).

Unless it is viewed against the broad perspective of ecology, bioclimatology cannot be clearly understood. Ecology deals with the complex interactions among organism, function and environment. Bioclimatology is "the study of the direct and indirect interrelations between the geophysical and geochemical environment and living organisms, plants, animals, and man" (8). The bioclimatologist then is primarily concerned with the atmospheric environment and its affects on the organism. In the context of this symposium the organism of chief interest is man.

The work of the human bioclimatologist follows two broad avenues. In the one instance, the questions are submitted to investigation in a controlled environment: a climatic chamber, a hot room, a cold room. Here the physical environment can be rigidly controlled and the reactions can be explored by the most precise physiological techniques. One or more environmental factors can be varied at a time and frequently the observations can be expressed in equations. In the other instance, the questions are submitted to investigation in the natural atmospheric environment, i.e.

[1] Joint with the Committee of Bioclimatology, American Meteorological Society.

[2] President, International Society of Bioclimatology and Biometeorology.

the weather. Controlled conditions are more difficult to achieve. One frequently has to employ complex statistical procedures to bring out weather effects. The results of studies made in controlled environments may not hold up. The predictions from these studies must, however, be tested in nature for man does not live in a climatic chamber. But more important, the bioclimatologist who studies weather must have an ecological point of view in order properly to evaluate and interpret his observations. His facts must be scrutinized in such a way that the impact of the total environment is evaluated. Only from this viewpoint can he realistically assign relative significance to weather. Only within this broader framework can his investigations have practical value for the solution of problems involving betterment of man's health and welfare.

Unfortunately much of human bioclimatology has not been given such an integrated evaluation. The facts have been marshalled to support various theories of climatic determinism (9). The Study Group on Climate and Economic Development in the Tropics of the Council on Foreign Relations (10), in contrast, has recently demonstrated the value of the integrated approach in appraising the complex problem of the lack of socio-economic development in hot humid regions.

To achieve the necessary background for an integrated attack on bioclimatological problems, a synthesis of known facts must be made, the significant problems must be delineated and an active collaboration must be brought into being among investigators having a mutual interest in the effects of the environment on man and other living organisms. To make such a program vital and enduring we must also educate medical students and graduate students in the medical and biological sciences so that they will think holistically, so that they will recognize the environment as a significant factor in etiology, therapeutics and prognosis. The prospectus thus must include 'broad training' and 'integrative thinking'—generalization not specialization.

For many decades work in bioclimatology has been conducted by isolated individuals. In Europe some of these men founded schools of thought. Dorno of Davos and Linke of Frankfurt are notable examples; they and their students have made lasting contributions. Over the years there have been a large number of foreign bioclimatological societies, conferences, research institutes and university training programs. For example, the Tenth International Congress on Thalassotherapy and Hydroclimatology was held in April at Cannes, France; the Eleventh International Meeting on Alpine Climatology will be held in May in Saint Vincent, Italy; and the Second International Congress on Social Climatology will be held in Briançon, France, in June. The only technical journals devoted solely to bioclimatology are edited and published in Europe.

In North America there have been no schools of thought in the European tradition and, until recently, few bioclimatological societies, conferences and university training programs. The American Climatological and Clinical Association was founded in 1884, 'for the study of Climatology, Hydrology, and Diseases of the Circulatory and Respiratory Organs' (11). It no longer exists. When the American Meteorological Society was organized in 1920, its original list of committees included a Committee on Physiological Meteorology with Ellsworth Huntington as chairman (12). The Committee survived but a few years. More recently a *Journal of Human Ecology* was published. Its life was three years (1951–1953).

In 1950 the American Meteorological Society created a Committee on Bioclimatology (13). Its chief objectives have been to further the growth of bioclimatology and to formulate standards for graduate training. It has sponsored three sessions on bioclimatology at national meetings of meteorologists (14–16), has edited and published a monograph (17) and has established an award for meritorious research (18). The present conference represents an effort initiated by the Committee's present chairman, Dr. Konrad Buettner.

In 1956 the Maricopa County Medical Society of Phoenix, Arizona, sponsored the organization of an 'Institute of Medical Climatology'. The aim of this group of physicians is to obtain basic information on climate and health and on climatotherapy.[3] This development is significant, for an urgent need of bioclimatology is a critical *re-evaluation* of the idea that climate plays an important role in the treatment of disease.

Physicians, such as Solley (20) and Loomis (21), in the past recommended that medical students be taught medical climatology and climato-

[3] In 1933 H. H. Kimball of Blue Hill Meteorological Observatory made a similar proposal for establishing a 'physiological meteorological station' (19). In his article he points out that the American Climatological and Clinical Association was also planning a climatotherapeutic institute to be located in Tucson, Arizona. To my knowledge these early proposals never materialized.

therapy. To my knowledge no American medical school now has such a program. At the University of Illinois in Urbana and at the University of Washington graduate curricula are developing in bioclimatology. The Committee on Bioclimatology has prepared a report on 'Graduate Training in Bioclimatology' (22) which it plans to publish in the near future. In a subsequent report this committee will discuss the results of an international survey of graduate training in this field which is now being conducted.

Medical educational philosophy in America has been changing in recent years. We might characterize the trend as a return to the Coan concept of man-environment. Students are now receiving instruction in 'human ecology' (23). They are being taught that the 'patient as a whole' is the important point of view in medical practice, that the patient cannot be considered as isolated from his environment. Some of the new courses even include discussion of bioclimatology. This trend is a most encouraging one, for it indicates an effort to give an integrative training to future generations of medical practitioners and investigators.

It was the regional growth of interest in bioclimatology which led to the creation of the International Society of Bioclimatology and Biometeorology. The inaugural meetings held in Paris, France, in August 1956, were most successful (8). The first scientific congress will be held in Vienna in September. Membership in the Society now numbers well over 400. Almost every branch of meteorological, biological and medical science is represented. The basic goals of the Society are to encourage and stimulate the tremendous integrative job required in making a critical evaluation of all areas of bioclimatology and to promote sound investigation of the really significant problems delineated by the synthesis. These goals shall be implemented by holding international symposia and congresses, creating an international clearing house, creating an international journal, forming technical committees to investigate basic problems and fostering the development of national groups in bioclimatology and related sciences and their international cooperation.

The required integrative work can, I think, best be launched by national and international groups gathered to discuss a common question. Although the ultimate synthesis may have to be made by one or more qualified individuals, the preliminary collation lends itself admirably to a cooperative undertaking by a group. The cooperative effort basically involves a working together of meteorologists and biological and medical scientists.

There have been, in the past, numerous pleas for such cooperative effort by physicians and others (24, 25) but only recently has a serious effort been initiated in this direction by the U. S. Weather Bureau. Just a few months ago Dr. Helmut Landsberg, Director of the Office of Climatology, established a Section on Human Bioclimatology (personal communication). The task of the Section will be primarily to advise and answer queries received by the Weather Bureau on matters of bioclimatology. The Section could ultimately assume an important job in giving to meteorologists employed in regional offices basic information on bioclimatology. These meteorologists are constantly being asked for a wide variety of information ranging from opinions of weather effects on perishable commodities to advice on the most suitable climatic conditions for residence of patients with particular types of acute or chronic disease. Bioclimatologists should work closely with the Weather Bureau to keep the personnel informed on the current status of knowledge and to assist in developing any required educational programs.

A final, and perhaps most important, problem must be considered. There is an increasing demand in many areas for persons trained in the principles and technics of bioclimatology. Dr. D. H. K. Lee and I have recently discussed this matter and I should like to summarize our thoughts for your consideration.

Three possible solutions come to mind. First, an Institute of Bioclimatology might be created. The most effective type of institute would be a privately endowed institution located on the campus of a major university. The advantages of such an institute would be that duplication of costly physical facilities could be avoided and that having a group of men working together might be mutually stimulating and provoke a greater output of scholarly investigation. There are, however, two disadvantages. The continued productive output of such an organization generally depends upon the stimulating effect one man has on his colleagues. When he leaves, as history teaches, the institute frequently deteriorates. A more important disadvantage is the fact that a great deal of capital has already been invested in the basic environmental physical plant at several universities and governmental laboratories. It is

our thought that the solution does not lie so much in the creation of a new institute as in the more effective utilization of that which is already available.

The alternative plans involve use of existing facilities. A Study Group, such as the Committee on Bioclimatology, could first make an inventory of the equipment and the manner in which it is used for research and for graduate training. Second, the available courses and training programs could be documented. Then, a series of curricula could be prepared integrating the various courses into an effective training program. Each of us in bioclimatology has something different to contribute toward the instruction of students both in point of view and in problems under study as well as in available equipment.

Students could apply for graduate training either at the pre-doctoral level or at the post-doctoral level. Pre-doctoral students would take their basic work in the institution of their choice. With the assistance of the Study Group, they could plan to spend portions of one year at other institutions so as to obtain advanced training in the phases of bioclimatology that would be most useful to them. It might even be that some students would conduct their graduate research at some institution other than the one ultimately granting the doctoral degree. The thesis research should be conducted where the most appropriate facilities were located.

Two basic problems that face this pre-doctoral plan are financial and academic. Travel grants should be available for qualified students to provide support during their period of advanced training. The Study Group would have to work out the means whereby graduate credit for the courses taken in different institutions could be applied toward a doctoral degree without unduly prolonging the time required to fulfill the academic requirements for graduation.

To avoid the complexities of satisfying inter-university course requirements, the most feasible plan might be to establish post-doctoral traineeships in bioclimatology. Qualified students with doctorate degrees could apply for such traineeships and, with advice from the Study Group, they could take special didactic and laboratory work at the institution best equipped to meet their needs. The grants for the post-doctoral students should be administered by the Study Group.

Through either the pre- or the post-doctoral programs, the joint talent of the bioclimatologists in this country and the available equipment could be utilized. Students admitted to either program should be carefully selected and critically examined prior to beginning advanced training and research. For the pre-doctoral candidate, we do not propose that a Ph.D. in bioclimatology be created. In fact, at present, we (26, 27) are strongly against such a degree. The students should be working toward a doctorate in one of the basic biological or medical sciences or geography. The bioclimatological program would provide the minor. The objective of the graduate training, in either case, should be to provide the student with a broad background so that he could continue to make substantial contributions.

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AIM AND PURPOSE OF SYMPOSIUM ON BIOCLIMATOLOGY[1]

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THE WORD bioclimatology was coined, around 1910, by Carl Dorno in Davos, Switzerland, the place of Mann's famous novel *Magic Mountain.* This place was and still is sought for its climate by persons with tuberculosis. We shall devote this Symposium to renewing study of the effects of climatic environment on man. The emphasis of such studies lies in the interplay between climatology and human biology, not in these fields themselves. We therefore consider ourselves fortunate to have members of two well-recognized societies, namely the American Physiological Society and the American Meteorological Society, here for a combined meeting. The aim of this Symposium, then is to combine existing groups for joint discussion instead of creating one more specialized group. Dr. Sargent has already mentioned the organizational aspect of bioclimatology.

Bioclimatology describes all effects of the natural environment on living organisms; this symposium is restricted to the human aspect. The word encompasses climatophysiology, climatopathology, meteorobiology, the effects of certain orographic factors and cosmic effects, if any. To avoid the dispute over 'meteorology' and 'climatology', we shall use the word 'geophysics' to indicate 'natural physical environment'.

Emphasis should be placed here on those researches which need it rather than on those already enjoying organized cooperation such as aviation and space medicine. Bioclimatology is a borderline science and becomes more important to us the more it investigates the border area rather than the main fields on either side of the border. Recording room temperature during surgery is not bioclimatology yet, but a treatise on the climate of a 'climatic resort spot' is not either, unless it is accompanied by proof of why these climatic elements are important to human health.

Bioclimatological research can be subdivided into three phases of approach.

a) *Quantitative Bioclimatology* deals with measurable changes in man caused by measured environmental elements in a way which is understandable from the physical, chemical and biological viewpoints. The essential bioclimatic element in this search is generally the physical transfer mechanism. This phase may therefore also be called physical bioclimatology. Examples are thermal effects and solar ultraviolet effects.

b) *Qualitative Bioclimatology* describes observed medical facts obviously caused or triggered by atmospheric agents. There are no clear-cut, quantitative relations between the agent and the illness or the effect. Most agents are aerosols or air pollutants, and the most striking effects are allergies. Effects of smog and radioactive products might belong in this category.

c) *Statistical Bioclimatology* searches for correlations between any number of weather elements or weather accords and human actions, reactions and health. The causative agent is generally not known and, in many cases, is not even suspected. This is, of course, the infant stage of research. No item of investigation can remain too long in this stage, but must advance to the higher stage (*a* and *b*), be recognized as not bioclimatic at all, or be abandoned as a pseudoproblem.

Any effect of *group a* is, of course, wholly reproducible in the laboratory and those in *group b* are at least qualitatively reproducible. Surveys concerning some phases of each of these fields will be offered in this Symposium. A few words, however, should be devoted to those fields of knowledge not discussed.

One recurring question is that about a 'healthful' climate, a question which may be countered by the question, 'healthful for whom and when and for how long?' Progress by technicians and hygienists has made more and more areas habitable by providing food, water and communication and by preventing most infectious diseases. Protection from climatic thermal hardships has progressed from the lean-to to the air-conditioned house, and can be had everywhere for a price. However, a large migration to thermally comfortable climates has begun and will increase.

[1] Contribution No. 26 of the Dept. of Meteorology and Climatology, Univ. of Washington.

Ailing people and their physicians search for a suitable outdoor climate. Advice is frequently given from rather generalized biological statements, combined with scanty climatological knowledge. Physicians are taught to use every treatment from penicillin to the scalpel, except the omnipresent climate and weather—or protection from it. They might be amazed to learn that the lower Rio Grande Valley in July equals in moist heat Devil's Island; that summer climates of the Southeast and of west Texas are fundamentally different; that in a northern part of the Midwest a heated house in winter is much drier than an Arizona desert, a fact of interest to dermatologists since the stratum corneum cracks when dry; that Santa Monica has fog and Los Angeles smog; that the much publicized death rates during heat waves in eastern states have no parallel on the Gulf Coast, where the temperature and the water content of air are always higher; that every environment can be endured for a specific time; that the reduced oxygen partial pressure may be responsible for many automobile accidents at moderate altitudes.

A temporary change of climate is sometimes recommended; it frequently helps. A vacation nearly always does. Many records exist showing how man's physiological, psychological and clinical parameters change during a four-week stay at the sea coast, in the mountains or in other climates new to the patient or test subject. These changes could be caused by cooling power, solar ultraviolet, altitude, inhaled ocean salts and other climatic factors; such changes could also stem from exercise, mental relaxation, regular life and other vacation factors.

Since climatic and vacation elements mix, separate evaluation of them becomes difficult. I once attended a series of speeches which declared that, for nearly everybody, the climate of the North Sea and of the lower mountains and of the higher mountains and of the sultry Adriatic Coast is healthful. Since all are good and since a smog-free home town has a climate too, we may well conclude that a vacation is a good idea, and that except for a few recognized medical counter indications, we know little about 'healthful' climates. A new, valuable method to test effects of climatic cures has been developed by H. Jungmann in Hamburg. This test is based on circulatory measurements.

There are, of course, 'bad' climates, which I shall mention briefly together with a few more bioclimatic problems which cannot be discussed in detail.

a) *Thermal extremes* such as an Arctic winter or the Gulf Coast summer cannot be recommended. Our respiratory organs are sensitive to both temperature and humidity. The problem seems difficult as demonstrated by the frustrations in the research on common colds. Much work must still be done to differentiate the effects of drying out of membranes, of cooling or heating of upper respiratory organs, of subsequent influences on the blood supply to the membranes, and of a change in the number, kind and activity of microorganisms and viruses and in their mode of transfer. Noxious pollutants from natural (forest fire, volcano) and artificial sources obviously make a bad climate. So do microorganisms and pests, especially in tropical areas. We shall hear more about allergies later.

b) *Nonthermal effects of solar light.* Solar ultraviolet causes reproducible and measurable skin reddening, immediate pigment, late pigment and, sometimes, skin cancer. Playing in the sun with minimum clothes is fine; but is the solar spectrum responsible for the beneficial effects, besides its influence on temperature, humidity, visibility, the individual's mood and exercise?

c) *Very extreme climatic situations* from an Arctic blizzard to a fire can be endured for a limited time. Endurance time for a protected or exposed body is the only comparable measure for total climatic strain. Heat of fire can be a thoroughly natural element, e.g. a forest fire from lightning.

d) *A resort spot should watch its microclimate.* Valleys tend to collect smog and cold air on clear nights, dry surfaces prevent allergens from settling, especially in windy weather. West-facing picture windows cause an unnecessary solar heat load.

e) *Spas and baths claim all kinds of cures.* Many of them are subject to the same argument of vacation versus environmental effects mentioned above. The science of spas is venerable and thousands of years old. But when the author recently asked men in this field, in this country and abroad, whether water passes into and through the human skin, they did not know. Well, water passes.

The aim of this Symposium, then, is to serve as a nucleus for the condensation, combination and integration of the two forces, environmental and biological, which decide whether we like a weather or not. This cooperation seems quite necessary in view of the many exposures of man to climatic extremes in expeditions, in the Armed Forces and in everyday life.

THERMAL STRESS AND PHYSIOLOGICAL STRAIN[1]

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ONLY the major considerations in the evaluation of environmental stress and the resulting physiological strain are considered in this report. While there are many additional considerations which may be brought to bear on specific problems, the basic considerations outlined here underlie every problem. Unfortunately, one or more of these considerations has been neglected in most investigations, oversights that detract from the value of the work and prevent comparison of various studies. It is hoped that the simplicity of this report will direct attention to the essential elements common to all such investigations.

These considerations are not new nor did we discover them; only the simplifications and emphasis are ours. The names of Burton (1); Buettner (2); Winslow, Gagge and Herrington (3); Blockley, McCutchan and Taylor (4); Belding and Hatch (5); Hardy and DuBois (6); and Wezler (7) are familiar to those of you who work in this field.

Stress is defined as the tendency of the environment to cause a change in the individual. In the cold, the stress is a combination of air temperature, wind and radiation. In the hot environment, the stress is a combination of air temperature, wind, vapor pressure and radiation.

In the cold, these combined elements give rise to a large range in thermal stress. On a recent trip to Alaska, Alan Burton suggested that the Eskimos must have a series of words to describe the Arctic temperatures but, alas, investigation proved that the Eskimo has only the word *uqsaluktug*, it is cold. He can say, *uqsaluknitug*, it is not cold, or *uqsalukpatug*, it is very cold, or *alapaa* which is best translated 'Br-r-r-r.' The Eskimo is not as original in his weather description as the Navajo who has a male rain (a narrow, fast moving storm with outriggers of thunder and lightning) and a female rain (slowly wetting, a mild, effective blessing).

Strain is defined as the change in the homeotherm brought about by the stress—the alteration in physiological function to maintain temperature and the degree to which this change is effective. If the approximations of Gagge, Burton and Bazett (8) for the human body are used,

Heat loss to the environment

$$= K_0(T_s - Ta) = H$$

where, T_s is the average skin temperature; Ta is air temperature (qualified according to air movement); K_0 is equal to $h_r + h_s$ (the heat transfer coefficient for radiation and for convection); and H is $\overline{H}$—He in steady state (He in non-sweating state, i.e. below a skin temperature of 33°C, is assumed to be $0.24\overline{H}$). $\overline{H}$ is total body heat loss.

When H at a steady state with a fixed heat production and standard clothing is considered,

$$H = \frac{1}{0.32}(T_s - Ta)$$

when H equals 38 Kcal/m²/hr; T_s equals 33°C; Ta equals 21°C; v equals 20 ft/min; and 0.32 equals the insulation of air, I_a (0.14), plus that of clothing, I_{cl} (0.18). Conditions which cool the body exist when the Ta is less than 21°C.

Figure 1, taken from Burton (1), illustrates insulation in clo units at various temperatures and heat productions (where M or Met is 38 Kcal/m²/hr).

Air movement serves to reduce I_a

$$H = \frac{T_s - Ta}{I_{cl} + I_{sa} - W}$$

when I_{sa} is the insulation by still air, and W is the wind decrement.

$$W = I_{sa}\frac{I_{sa}\beta\sqrt{v/v_0}}{1 + I_{sa}\beta\sqrt{v/v_0}}$$

where β[2] equals 12 Kcal/m²/hr/degree;[2] v is the

[1] Supported in part by the United States Air Force under Contract AF 18(600)-1467.

[2] β is 24 Kcal/m²/hr/degree if the geometrical surface instead of the heat-losing surface is considered.

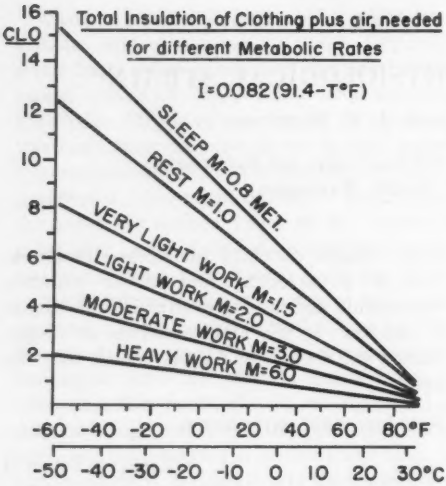

Total Insulation, of Clothing plus air, needed
for different Metabolic Rates
I=0.082(91.4-T°F)
SLEEP M=0.8 MET.
REST M=1.0
VERY LIGHT WORK M=1.5
LIGHT WORK M=2.0
MODERATE WORK M=3.0
HEAVY WORK M=6.0
CLO
-60 -40 -20 0 20 40 60 80°F
-50 -40 -30 -20 -10 0 10 20 30°C

FIG. 1. Total insulation ($I_{cl} + I_a$) for different environmental temperatures and metabolic rates. For the insulation of clothing required (I_{cl}), the insulation of air (I_a), dependent on wind, must be subtracted from the total insulation. (From Burton, 1.)

velocity in m/sec; and v_0 equals 1 m/sec in reclining supine man. By rearrangement,

$$H = \frac{T_s - (Ta - HW)}{I_{cl} + I_{sa}}.$$

As I_{cl} becomes large, the significance of the wind decrement decreases. Thus, with the standard clothing value, the total insulation could change by 45% as the wind increased; while with four times the standard clothing, the change would not exceed 16%. Figure 2, a further example from Burton (1), illustrates the wind decrement for various wind speeds at three levels of metabolism.

To continue the analysis of environmental stress as it has been presented by Burton, the final consideration at air temperatures below 21°C involves the heat gain from solar radiation (R). This heat will be added at the clothing surface:

$$H = \frac{T_s - T_{cl}}{I_{cl}} \qquad H + R = \frac{T_{cl} - Ta}{I_a}$$

$$H = \frac{T_s - (Ta + RI_a)}{I_{cl} + I_a}$$

Figure 3 illustrates the radiation increment for different cloudiness, clothing colors and wind speeds. As I_{cl} increases, the addition from radiation will also decrease in significance. Thus, a statement of the environmental stress at temperatures below 21°C should include: air temperature, wind decrement, and radiation increment.

Numerous methods of combining these factors such as température resultante, operative temperature, cooling power, standard operative temperature and cooling temperature have been summarized by Buettner (2). Siple's (9) wind chill index has limitations; its successful feature has been aptly assessed by Burton (1) in reference to the exposed face. The mechanisms available for maintenance of heat balance with negative heat load are: increased heat production and added insulation.

Departure from the steady state will be evidenced by: *1*) an increased heat production, *2*) a decreased skin temperature and *3*) a decreased

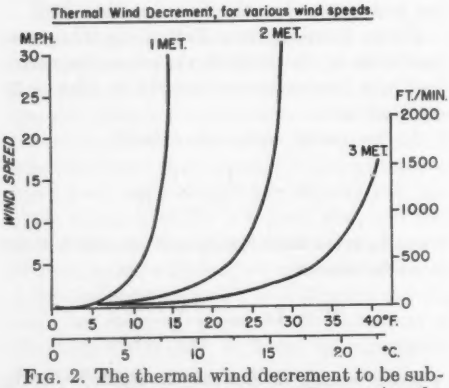


FIG. 2. The thermal wind decrement to be subtracted from the thermometer reading to give the equivalent still-air temperature. (From Burton, 1.)

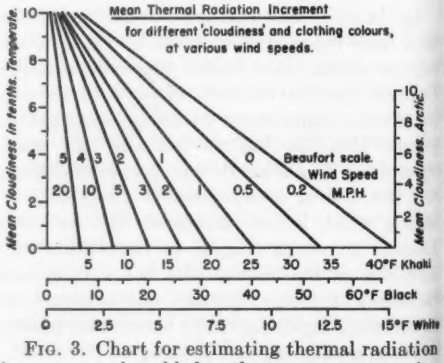
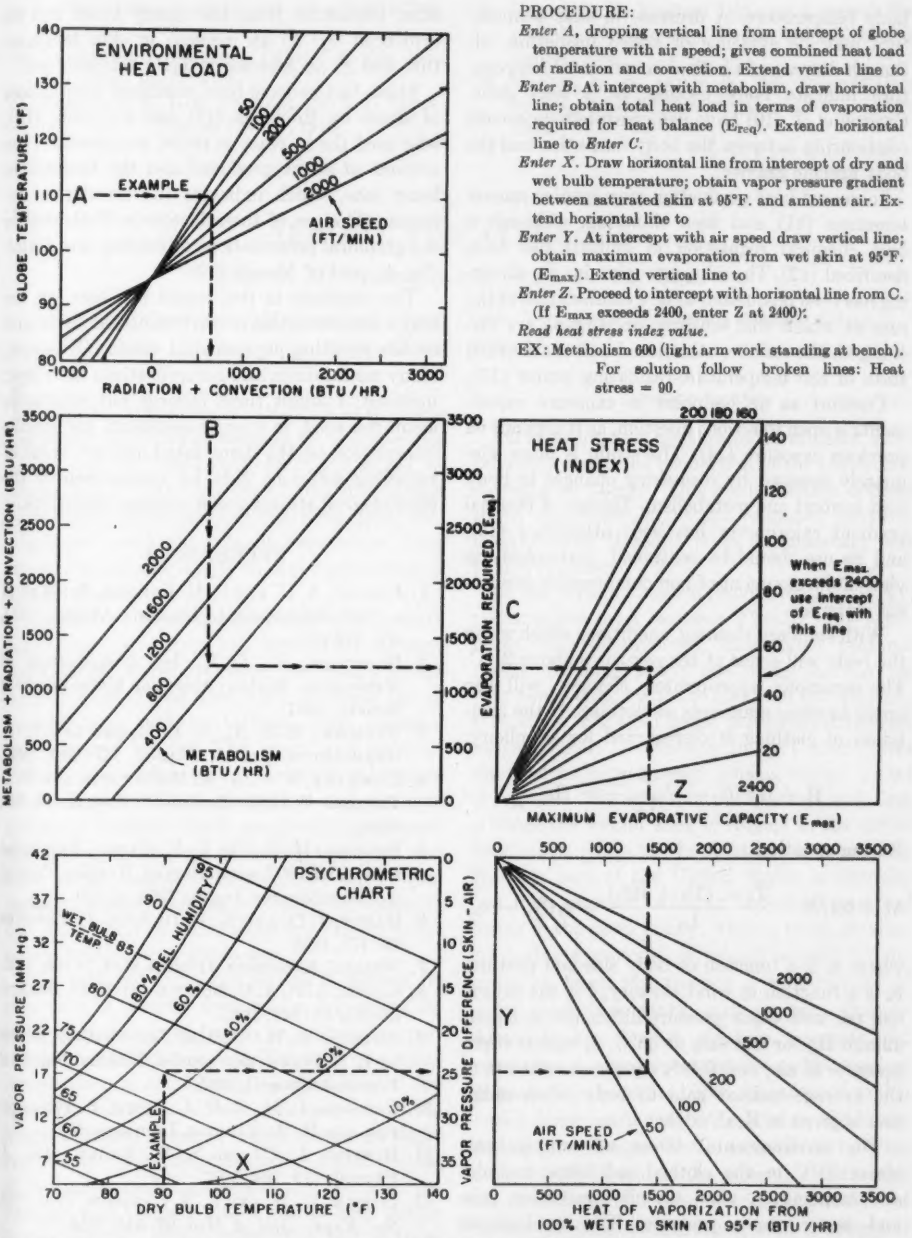

Mean Thermal Radiation Increment
for different 'cloudiness' and clothing colours,
at various wind speeds.
Mean Cloudiness in tenths. Temperate.
Mean Cloudiness. Arctic.
Beaufort scale.
Wind Speed
M.P.H.
0 5 10 15 20 25 30 35 40°F Khaki
0 10 20 30 40 50 60°F Black
0 2.5 5 7.5 10 12.5 15°F White

FIG. 3. Chart for estimating thermal radiation increment to be added to the temperature to give the equivalent shade-temperature. (From Burton, 1.)



PROCEDURE:

Enter A, dropping vertical line from intercept of globe temperature with air speed; gives combined heat load of radiation and convection. Extend vertical line to

Enter B. At intercept with metabolism, draw horizontal line; obtain total heat load in terms of evaporation required for heat balance (E_{req}). Extend horizontal line to *Enter C*.

Enter X. Draw horizontal line from intercept of dry and wet bulb temperature; obtain vapor pressure gradient between saturated skin at 95°F. and ambient air. Extend horizontal line to

Enter Y. At intercept with air speed draw vertical line; obtain maximum evaporation from wet skin at 95°F. (E_{max}). Extend vertical line to

Enter Z. Proceed to intercept with horizontal line from C. (If E_{max} exceeds 2400, enter Z at 2400):

Read heat stress index value.

EX: Metabolism 600 (light arm work standing at bench). For solution follow broken lines: Heat stress = 90.

This index represents a rational approach to evaluation of heat stress in terms of: *1.* thermal load which is imposed on a standard man; *2.* capacity of the environment to accept the load; and *3.* physiological capacity to meet the demands over a period of 8 hours.

The standard man represents a composite of young, fit, acclimatized men who have been subjects of physiological investigation of the effects of heat in the laboratory and in the field.

FIG. 4. Flow charts for determining heat stress index values. Example: globe 110, dry bulb 90, wet bulb 75, air speed 100, metabolism 600. For solution, follow broken lines: Heat stress = 90. (From Belding and Hatch, 5.)

body temperature. A decrease in skin temperature may be evidence of added insulation, although decreases in both skin and rectal temperature indicate body cooling, or a heat debt. Carlson *et al.* (10) have demonstrated an inverse relationship between the body insulation and the body specific gravity.

Increased heat production may involve muscle tenseness (11) and then shivering, although a true chemical regulation in animals has been described (12). The adequate stimulus for shivering has been postulated to be a combination of the rate at which cold-sensitive end-organs are discharged, the number stimulated and the thermal state of the temperature-regulating center (13).

Comfort as an endpoint in exposure experiments is open to serious question, as it depends on previous exposure (14). The strain is more adequately assessed by measuring changes in body heat content and metabolism. The use of thermal gradient calorimetry has been attempted (15), and its use should be continued, particularly in view of development of non-compressible insulating materials.

With standard clothing, conditions which warm the body will occur at temperatures above 21°C. The equations, appropriately changed, will also apply to other standards of clothing. If the insulation of clothing is disregarded for simplicity,

$$\overline{H} + 0.15R - \frac{T_s - Ta}{I_{sa}} = He$$

During sweating,

$$M + 0.15R - \frac{T_s - (Ta + \overline{H}\phi_1)}{I_{sa}} = \phi_2 f(e_s - e_a)$$

where ϕ_1 is a function of body size and posture; ϕ_2 is a function of wind velocity; f is the caloric loss per unit vapor pressure difference; e_s equals 42 mm Hg for wet skin at 35°C; e_a equals vapor pressure of air; and 0.15R equals an estimate of the average radiant gain to body when radiation is given in Kcal/m²/hr.

The environmental stress at temperatures above 21°C in the clothed individual include: air temperature, wind velocity, radiation gain and water vapor pressure. The mechanisms available for maintenance of heat balance are increased circulation to the skin and evaporation. Buettner's work (16) emphasizes the fact that sweat and diffusion are combined in net water loss, the amount of diffusion being influenced by vapor pressure or the wetness of the skin. Departure from the steady state will be evidenced by: *1*) an increase in skin temperature and *2*) an increase in body temperature.

These factors have been combined into indices of strain by Robinson (17) and by Craig (18), who used the increase in rectal temperature, the amount of sweat produced and the increase in heart rate, which indicates the circulatory response. The use of these factors is illustrated in the graphical presentations of Belding and Hatch (fig. 4), and of Mauch (19).

The emphasis in this report has been on the major considerations of environmental stress and on the resulting physiological strain. Of course, many assumptions and generalizations have been included; I admit these hazards but emphasize again the need, in every experiment, for specific information on the items listed and the value of recording sufficient data for comparison of the physiological strains under various conditions.

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AEROSOLS AS ALLERGENS

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IN THIS PRESENTATION we shall not only accept the broad definition of aerosols as 'a suspension of fine solid or liquid particles in air or gas, as smoke, fog or mist,' but shall broaden it further to include larger particles such as pollen, dust etc., which can reach our mucous membranes. How important are such inhalants in the production of allergic disease?

About 7 or 8 million people in this country suffer every year from hay fever. This not only produces much inconvenience, but results in millions of days of loss from work or school and in complications such as asthma, sinus infection and impaired hearing. The season and its duration vary depending on the variety of substances to which the sufferer is sensitive and the geographical location. Such symptoms are caused mainly by the pollens of ragweed, grasses and other plants, fungus spores and insect dusts. Nonseasonal allergic rhinitis is also quite common, and about half of such cases are known to be caused by air-borne substances.

Less frequent but more serious is asthma. It may vary in severity from minor, occasional attacks of shortness of breath to frequent or persistent and disabling disease. The most important complication is emphysema—a stretching of the lung sacs and a loss of the elastic property of the lungs. This may result in breathlessness on slight exertion and permanent disability. Asthma is one of the most disabling diseases and we must remember that we have 2 to 3 million such people in this country, and in more than half of these inhalant allergens can be demonstrated as a major cause.

Other diseases which may be produced by air-borne allergens are conjunctivitis and atopic dermatitis.

SPECIFIC CAUSES

On exposure and re-exposure to inhalant allergens only a selected proportion of the population develops an allergy. This predisposition appears to be dependent on as yet unknown constitutional factors. Heredity plays an important part in this tendency.

Of the inhalant agents responsible for allergy, pollen is the most frequent. Usually only wind-borne pollen is involved, since insect vectored pollens are large, heavy and sticky and only on very close contact with the plant can they produce symptoms. The wind-borne pollens are in the size range of about 10–20 micra. In the United States the most important pollen causing allergy is that of ragweed. The season in the Midwest and East is about the second week of August to the end of September, while in the South it is later. The Western part of the United States is virtually free from ragweed. Another important group of plants is the grass family, while a third, producing spring symptoms, is usually caused by the pollen of trees. But there is a wide variety of pollen-producing plants, such as sage, Russian thistle, kochia, English plantain, mesquite and many others, which determine the pollen problem in particular localities. The amounts of pollen have a large bearing on the incidence and intensity of the allergy. These amounts vary with many factors, such as the general climate and soil conditions, the pre-flowering abundance of rainfall, sunshine, temperature, wind velocity and perhaps other factors.

Up until about 25 years ago molds as causes of asthma and hay fever were regarded as medical curiosities. At about that time our own observations and those of some others indicated that the

air-borne spores of the common soil fungi are responsible for a large number of the respiratory allergies. Subsequent experience has amply confirmed this concept and it is now generally agreed that mold spores are next to pollen in importance as causes of asthma and hay fever. The molds which give rise to these spores are common soil fungi which live particularly on grains and grasses and in the soil where such plants grow. Alternaria and hormodendrum are perhaps the most important, but there are many other fungi which must be considered. Most of these spores vary in size from about 2–50 micra. The Midwest, bread basket of this country, is the most important area for this type of mold allergy. No location can be said to be free of molds, but the dry areas and the high altitudes have negligible amounts. Despite the high temperatures or humidity of certain areas, such as the extreme South or the West Coast, mold allergy is much less frequent there than in the Midwest, apparently due to the fact that the plant life indigenous to these areas does not support the type of fungi which are apt to sensitize.

The stings of insects have been frequently known to produce severe and even fatal allergic reactions. The first instance of such a reaction was recorded in 2641 B. C. when, according to hieroglyphics found at his tomb, King Menes of Egypt was stung to death by a wasp or hornet. The scales, hairs and dusts of special insects in special environments or occupations have been reported as causes of asthma and hay fever. Recently we have presented evidence that insect dusts are common causes of allergy and that the origin of this dust is probably from the debris of disintegrated insect life in the top soil and vegetation rather than from the living insect in the air. This is a relatively new field and much work needs to be done to develop it.

Thus far we have spoken chiefly about seasonal allergy. There is a host of substances non-seasonal in nature, producing similar effects. The dandruffs from a number of animals which commonly produce allergy are those of the dog, cat and horse. Among medical students and research workers we have seen many instances of allergy to the dandruff of guinea pigs, rats, mice and rabbits. Other animals have also been known to produce allergy. Many people with asthma and rhinitis react to house dust. The actual ingredient in such dust responsible for the allergy has not been identified. It is known, however, when such organic material as cotton linters in the mattress and furniture ages a substance is produced which seems capable of acting as an allergen. Although the evidence is not yet complete it would appear that such 'house dust' antigens are more readily formed in certain climates, particularly where it is damp.

There are many other air-borne substances responsible for allergic symptoms. The list is too large to discuss and we shall only mention several representative ones here: starches in cosmetics; vegetable gums (Karaya, acacia, tragacanth, flaxweed, quinceseed) for hair-setting and other purposes; penicillin and streptomycin as sprays or from occupational exposures; castor bean dust as an air contaminant from its source of processing or from its use as a fertilizer; occupational exposures, such as from chlorinated compounds, dyes and many other chemicals. It is well known also that the odors or vapors from cooking or even raw foods may produce allergic symptoms. This is particularly true of such foods as fish and eggs. That this is not a matter of psychic suggestion we have demonstrated several years ago. Peas were cooked in an open utensil. A dish of salt solution was exposed several feet away. The salt solution was then sterilized by Berkfeld filtration. An intradermal injection (suitably controlled) of this solution gave a positive reaction in a patient who gave a history of asthma occurring while peas were being cooked.

SECONDARY AIR-BORNE CAUSES

In addition to the known specifically allergenic inhalant substances there are a number of conditions of the atmosphere which act as secondary irritants, producing the effect of further irritating the membranes so as to diminish their tolerance for the allergens. In some instances it has not been adequately ascertained whether such a cause is secondary or actually allergenic. Such well-known influences are soft coal smoke, smog, tobacco smoke, gasoline fumes, strong odors of any kind and irritating chemical fumes or dusts. Some of these irritants are parts of industrial wastes and general pollution of the atmosphere. It is not unusual for a suburbanite to start off from home feeling fine and be in the throes of an asthma attack by the time he reaches the heart of Chicago.

Mechanical irritation from dusts may produce similar effects. Dust storms in Arizona frequently aggravate respiratory allergies. Road dust will produce violent symptoms in the seasonal hay fever patient, while out of season it will cause no

more discomfort than in the non-allergic individual. Some of the effects of windstorms are due to the mechanical irritation from dust. The school teacher who has allergic rhinitis almost invariably attributes it to chalk dust. In no case have we been able to demonstrate an allergy to chalk, and it is quite obvious that this dust acts mechanically to irritate a nose allergically sensitive to something else.

The problem of air pollution by the activities of mankind is one of the least developed problems and is increasingly important to the welfare of man. It is for the purpose of discussing these factors and the effects of weather and climate that these symposia have been scheduled.

MANAGEMENT OF ALLERGY FROM AIR-BORNE SUBSTANCES

To complete the picture of the medical problem before us as a result of air-borne causes of allergy we shall briefly outline the customary management of these situations. For the temporary relief of attacks of asthma or allergic rhinitis we depend on such drugs as bronchodilators (aminophylline, isopropylepinephrine), sympathomimetics (epinephrine, ephedrine), antihistamines, iodides, cortisone, prednisone, ACTH and other drugs. Where the specific causes cannot be ascertained, such and similar drugs may constitute the only means of relief.

The specific management of allergy due to inhalants has one of two, and frequently both, objectives. Avoiding the allergenic substances is the most desirable goal. In some cases this can be accomplished with completeness and with a permanent relief of symptoms. Among such completely avoidable items are dogs, cats, feather pillows and occupational exposures. In the more common air-borne causes such elimination processes are not total. In such instances placing a barrier between the allergen and the patient may be of help. This includes such procedures as covering the mattress with a dust-proof casing, the use of window filters, complete air-conditioning of the home, clearing the air of particulate matter with the aid of electrostatic apparatus and the use of face masks.

The question of climatic changes and influence is a complex one. Some of the effects are known and predictable, others are obscure. Those who are allergic to specific pollens or molds can be steered to areas where these allergens are absent or markedly diminished. Dust allergy cases will probably have symptoms anywhere. A warm, or dry climate may have a salubrious effect on the mucous membranes in spite of the presence of the allergenic factor. Getting away from the country may produce improvement by diminishing the allergenic exposure. Moving to the country, on the other hand, may benefit the individual because he escapes irritants. Where infection is an important cause climatic changes may help. Other effects of climate are less definitive and may be perhaps the result of general improved health, getting away from the stresses and strains of work and domestic life, or may be purely suggestive. Our experience is that climatic changes must be approached rationally and at all times on a trial basis.

When inhalant allergens can be avoided only partially or not at all it is important to try to increase the individual's tolerance to them. The process of desensitization is a slow one and not perfect, but it is at the present time, next to elimination measures, the most important approach for lasting benefit in such allergies.

METEOROLOGICAL CONSIDERATIONS IN RAGWEED HAY FEVER RESEARCH

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THE PROBLEM of ragweed hay fever is an interesting research topic despite its complexity because of its apparent impact upon the American populace. It was estimated in 1940 that 2–4% of the entire population of the United States suffered from hay fever (1). In a 1938 survey of 1,000 families in a city of 35,000, it was found that 10% of the individuals had hay fever (2).

[1] Publication No. 2 on *Atmospheric Pollution by Aeroallergens* under research grant No. E-1379 (C) from the National Institute of Allergy and Infectious Diseases, Public Health Service.

In 1950 it was reported that the prevalence of hay fever had shown regular year by year increases (3). Thus it is conservative to say that there are today about five million hay fever sufferers in the United States. Not all of these are sensitive to ragweed pollen, but it is generally agreed that in this country, of all the cources of allergenic substances, ragweed is probably the greatest offender because of its ubiquitous nature, its prodigious production of pollen and the special adaptation by virtue of size, shape and density of its pollen grains for airborne dissemination.

Obviously, because of the mode of pollination of *Ambrosia*, meteorological implications enter the study of ragweed hay fever. We shall see that there are many additional points of meteorological interest in the total problem.

It is perhaps appropriate to consider for a moment what the total problem consists of. Briefly, it starts with germination of the *Ambrosia* seed, continues through pollen ripening and release, pollen transport, intake by the patient, the allergic response that is hay fever, and ends with the therapy employed. At any point of this 'seed-to-sneeze-to-treatment' sequence, a crucial finding could alleviate or even completely banish the disease of pollinosis. It is this fact, together with the evident importance of the disease, that justifies some allocation of monetary and scientific manpower resources for a comprehensive attack upon the problem. Meteorological factors appear to have significance at nearly all stages of development of the disease.

POLLEN PRODUCTION

Pollen may be considered as a crop much the same way as cereal grains or hay or vegetables are considered. It is a plant product that is contributed to by the health and vigor of the parent plant, and these in turn are subject to environmental factors which include the weather.

1) Distribution of *Ambrosia*. Perhaps the first consideration involving weather is that of the distribution of the plant. In great measure, the climate controls the geographic distribution of any particular plant species. In detail, the location and vigor of a particular plant is affected by its microclimate which is a product not only of the weather but also of the terrain, surrounding vegetation, type of soil, soil moisture and so forth. Although the general ecology of the *Ambrosias* is fairly well known, relatively few specific data on the microclimates associated with ragweed plant communities exist. Meteorological interest, therefore, enters the study of ragweed distribution in at least two regards, *a*) the gross over-all climatic determination of the ranges of various subspecies and *b*) the micrometeorological determination of the health, vigor and pollen productivity of individual plants.

2) Phenology. The relation of stages in the development of a plant to the weather conditions and events that affect it is called the phenology of the plant. In certain well-known cases (4, 5) in which the phenology of crop plants has been studied carefully, it has been possible to distribute harvest schedules in time by exerting appropriate control over the times of planting different fields. This is done on the basis of a knowledge of the response of the plants to the meteorological observables and a knowledge of the local climatology, or average behavior, of these observables. As the season advances, summation of the observed meteorological quantities in an appropriate manner yields increasingly precise predictions of the proper harvest time for each plot.

This same principle can be applied to the problem of determining any specified stage of development of a ragweed plant. It appears entirely possible that, for example, the stages of ragweed development at which herbicides would be most effective might be predicted very accurately by this means. Several other possibilities of opening an effective attack upon the pollen producers and of predicting for the benefit of preventive therapy are readily envisioned. Each calls for the use of advanced knowledge in at least one specialty such as plant ecology, herbicide chemistry, physiology or medicine in addition to meteorology. Herein lie the special dividends derivable from collaborations developed in team research.

3) Pollen Yield and Quality. To the extent that the phenological relationships are established for a plant and the relation of plant vigor to crop production is known, it is possible to apply weather data to pre-season estimations of crop character and yield (6).

Two distinct items of information appear to be pertinent. The first is the crop yield, which in the present case is the relative amount of ragweed pollen that will be produced. Suitable collaboration between meteorologists and botanists is leading to the development of predictors for this kind of information. One of the more difficult problems arises in obtaining a reliable measure of the total quantity of pollen produced.

The second item of information that might be

predicted successfully is the quality of the pollen crop, or in other words, its relative antigenicity per pollen grain. It is well known, for example, that the quality of wheat may be changed drastically by drought as contrasted to ample moisture. The literature contains subjective observations of an effect of this type upon ragweed pollen (7, 8). Good objective measures of antigenicity are required to make such observations completely satisfactory, but the possibility of establishing predictors is certainly present and interesting. Furthermore, in the process of arriving at such predictors, the knowledge gained about those factors that are significant in generating antigenicity in the pollen grains may well lead to preventive measures to be applied to the weed.

POLLEN TRANSPORT

It is not necessary to dwell upon the anemophilous, that is, wind pollinated, nature of the *Ambrosias*. It is by virtue of this characteristic primarily that meteorology enters the field of ragweed allergy. However, it may be appropriate here to point out some of the details, known and/or hypothetical, of the air-transport process.

1) Available Theory. Two distinct approaches have been developed theoretically, each of which has its special applicability. The first is the molecular diffusion theory approach put forward by Schmidt (9) and modified by Rombakis (10). The second is the statistical approach developed by Sutton (11, 12) on the basis of earlier theoretical studies by Taylor (13). This development has been contributed to by Bosanquet and Pearson (14) and more recently by Chamberlain (15). The salient features and the most immediately useful results of each theory are discussed below.

a) Schmidt-Rombakis theory. Schmidt (9) applied classical diffusion theory to derive equations for the probable vertical distribution and horizontal transport of the 'Luftplankton' (all the small particles of living matter that become airborne such as spores, pollens, seeds, insect eggs, bacteria and viruses).

The diffusion equation is:

$$\frac{ds}{dt} = \frac{A}{\rho}\frac{\partial^2 s}{\partial z^2} \qquad (1)$$

The solution to this equation gives the vertical distribution of s as a function of time, t:

$$s = \frac{Q}{\sqrt{\pi m t}}\, e^{-z^2/mt} \qquad (2)$$

In these equations,

Q is the number of pollen grains projected across level z_0 per unit area and unit time.

s is the number of pollen grains per unit mass of air at any height, z, above the source at time, $t > 0$.

A is the mass exchange coefficient, assumed constant for the present analysis.

ρ is the air density.

$m = 4\dfrac{A}{\rho}$.

$e = 2.71828$, the Napierian base.

It follows that the fraction, q/Q, of the released pollen grains that will be found above height z after time t is

$$q/Q = \tfrac{1}{2}\left\{1 - \Phi\left(\frac{z}{\sqrt{mt}}\right)\right\} \qquad (3)$$

in which Φ is the Gaussian Error Integral, the argument in this case being $u = z/\sqrt{mt}$

$$\Phi(u) = \frac{2}{\sqrt{\pi}}\int_0^u e^{-x^2}dx \qquad (4)$$

Introducing at this point the settling speed, c, of the particles, and considering that the air moves horizontally with a wind speed, v, carrying particles downstream, Schmidt finally derived expressions for the least flight duration, T, for the 1% of the particles that are carried farthest ($q/Q = .01$),

$$T = 2.7\frac{m}{c^2}; \qquad (5)$$

for the least flight distance, F, of the same portion of the particles,

$$F = vT = 2.7\frac{m}{c^2}v; \qquad (6)$$

and for the maximum height attained by the lowest flying of this portion of the particles, Z,

$$Z = 0.675\frac{m}{c} \qquad (7)$$

Rombakis modified the Schmidt derivations by adding a sedimentation term to the diffusion equation:

$$\frac{ds}{dt} = \frac{A}{\rho}\frac{\partial^2 s}{\partial z^2} + c\frac{\partial s}{\partial z} \qquad (8)$$

which provides a more valid initial approach. This results in the equation

$$q/Q = \frac{2}{\sqrt{ml}}\, e^{-c^2 t/m} \int_z^\infty e^{-(z^2/mt)-(2c/m)z}\, dz \qquad (9)$$

in place of *equation 3*. Defining a 'probable flight path' as that along which $q/Q = \frac{1}{2}$, that is to say, equal numbers of particles are distributed above and below this 'path', Rombakis transformed *equation 9* to an error function expression and derived expressions for the 'probable flight path'

$$Z = 0.4769\sqrt{mt} - ct: \qquad (10)$$

the 'probable flight distance', X,

$$X = 0.2275\, m\, \frac{v}{c^2}: \qquad (11)$$

the 'probable flight duration', τ,

$$\tau = 0.2275\, \frac{m}{c^2}: \qquad (12)$$

the 'maximum probable flight altitude', Z_m,

$$Z_m = 0.0569\, \frac{m}{c}: \qquad (13)$$

and the 'probable terminal speed of fall', γ,

$$\gamma = \frac{c}{2} \qquad (14)$$

On the basis of these results, Rombakis computed a table of values of X, Z_m and τ for nine different spores, pollens and seeds for two different values of m, and for five different wind speeds. A set of curves showing the probable flight paths of ragweed pollen grains (*Ambrosia elatior*) for various conditions is presented in figure 1.

It is clear that the form of Rombakis' solution is the same as that of Schmidt's. An additional

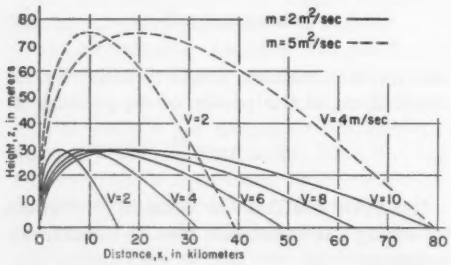

FIG. 1. Probable flight paths for ragweed pollen grains (*Ambrosia elatior*). Computed from *equation 10*.

contribution of Rombakis is the analysis of the problem using methods of statistical physics. This analysis leads independently to identical expressions.

A basic difficulty with this general approach is the fact that the eddy diffusion coefficient, $K = A/\rho = m/4$, varies through about 8 orders of magnitude (from 10^3 to 10^{11} cgs) in the atmosphere. In actual molecular diffusion processes, for which this theory has been proven adequate, the diffusion coefficient is much more nearly constant. One concludes, therefore, that molecular diffusion theory is less than adequate to explain eddy diffusion processes in the atmosphere, although as a first approximation and for general results such as those given above, it is useful. Accordingly, the statistical approach of Taylor has been developed in the expectation that it may more appropriately express atmospheric turbulence processes.

b) Statistical theory. Taylor (13) proposed that a process of mixing by eddy motions might be described statistically. If R_ξ is the correlation coefficient relating the initial velocity of a particle to its velocity ξ seconds later, and if the mean eddying energy does not vary with time during the period considered, then the scatter of a group of particles may be measured in terms of the standard deviation, σ, of their distances from an origin after T seconds:

$$\sigma^2 = 2\overline{u'^2} \int_0^T \int_0^t R_\xi\, d\xi\, dt \qquad (15)$$

In this expression, u' is the perturbation velocity defined by

$$u = \bar{u} + u' \qquad (16)$$

where u is the instantaneous wind velocity and $\bar{u}$ is the average wind velocity over some finite time period. It follows that over this same finite time period

$$u' = 0 \qquad (17)$$

Using dimensional analysis, Sutton (11) devised an explicit form of the correlation coefficient R_ξ in terms of the kinematic viscosity of air, ν, and the perturbation velocity,

$$R_\xi = \left(\frac{\nu}{\nu + \overline{u'^2}\xi}\right)^n \qquad (18)$$

In this relation, n is a positive number between 0 and 1 that depends upon the wind structure near the ground.

Sutton than proceeded to find functions that would satisfy the new form of *equation 15*:

$$\sigma^2 = 2\overline{u'^2}\int_0^T\int_0^t\left(\frac{\nu}{\nu+\overline{u'^2}\xi}\right)^n d\xi\,dt \qquad (19)$$

and at the same time would meet the boundary conditions and satisfy the continuity equation. The function expressing diffusion from a continuously emitting point source near the ground is

$$\chi(x,y,z) = \frac{2Q}{\pi C_y C_z \bar{u} x^{2-n}} \cdot\exp\left[-x^{n-2}\left(\frac{y^2}{C_y{}^2}+\frac{z^2}{C_z{}^2}\right)\right] \qquad (20)$$

in which χ is the concentration of material (dust, smoke, aerosol etc.) at the point (x, y, z); Q is the rate of emission of material from the source; and C_y and C_z are generalized diffusion coefficients in the cross wind and vertical directions, respectively.

Application of the statistical theory to problems of the spread of smoke and gases from industrial stacks was first developed by Bosanquet and Pearson (14). Sutton's more recent work (12) has incorporated some of the features of the Bosanquet and Pearson applications and has produced expressions that fit observational data somewhat better.

Chamberlain (15) has extended Sutton's theory to account for deposition. Introducing the concept of a velocity of deposition, v_g, which is defined as

$$v_g = \frac{\text{Amount deposited per cm}^2\text{ of surface each second}}{\text{Volumetric concentration per cm}^3\text{ above surface}}, \qquad (21)$$

Chamberlain derives an equation for the rate of deposition, ω, of particulate matter at the point (x, y):

$$\omega(x,y) = \frac{2Qv_g}{\pi C_y C_z \bar{u} x^{2-n}} \cdot\exp\left[-\frac{4v_g}{n\bar{u}C_z}\sqrt{\frac{x^n}{\pi}}\right]\exp\left[-\frac{y^2}{C_y{}^2x^{2-n}}\right]. \qquad (22)$$

The deposition rate on the 'plume' axis can be found from *equation 22* by setting $y = 0$.

The importance of the process of impaction of particulate material in promoting deposition is accounted for in a measure by the use of v_g. Of necessity v_g is evaluated from experimental data (table 1), which show in a general way that v_g increases with increasing wind speed. Referring to table 1, Gregory's experiment was done in a small wind tunnel whereas Chamberlain's was done outdoors. The agreement is quite good between these two very different experiments, but the wind tunnel (Gregory) apparently gives less consistent results at the lower wind speeds.

TABLE 1. VELOCITY OF DEPOSITION, V_g, FOR LYCOPODIUM SPORES (16μ DIAMETER) IN RELATION TO WIND SPEED

Gregory (16)	wind speed, u m/sec.	9.2	5.6	3.2	1.7	1.1
	v_g cm/sec..	2.3	1.3	0.2	0.3	0.7
Chamberlain (15)	u m/sec...	8.2		3.1	1.6	
	v_g cm/sec..	2.1		1.1	0.5	

Chamberlain points out that, as deposition proceeds from the lower portions of a cloud of airborne particulate matter, the vertical distribution of the material is modified and the total amount of material remaining airborne is depleted. Because the rate of deposition depends upon v_g, it turns out that at a given distance, x, from a source of variously sized particles, one particular particle (i.e. value of v_g) will give a maximum deposition rate, ω. The value of v_g that gives maximum deposition rate at distance x is designated v_{g^*}. The equation for v_{g^*} is found setting the derivative $d\omega/dv_g$ equal to zero:

$$v_{g^*} = \frac{nuC_z}{4}\sqrt{\frac{\pi}{x^n}} \qquad (23)$$

Using typical values: $n = 0.25$, $C_z = 0.12$, and taking $u = 5m$/sec the value of v_{g^*} corresponding to $x = 10$ km is $v_{g^*} = 2.0$ cm/sec. Thus, of all particles released to the air from a point source under the conditions stated, those contributing most heavily (in proportion to their initial number) to total deposition 10 km downwind would be the particles characterized by $v_g = 2.0$ cm/sec.

Chamberlain has also considered the effect of rain-washing of the particulate cloud. Essentially he has applied the Langmuir (17) collision efficiencies, $E(r, s)$, to determine the rate of removal, $\lambda(r, s)$ of particulates of radius r by raindrops of radius s:

$$\lambda(r, s) = \pi s^2 N(s) v(s) E(r, s)\, ds. \qquad (24)$$

The application of this equation requires knowledge of the drop size spectrum of the rain so that

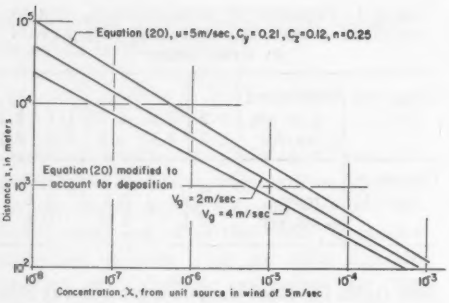
Equation (20), u=5m/sec, C_y=0.21, C_z=0.12, n=0.25
Equation (20) modified to account for deposition
V_g=2m/sec
V_g=4m/sec
Distance, x, in meters
Concentration, χ, from unit source in wind of 5m/sec

FIG. 2. Air concentration, χ at ground level directly downwind from ground level source as a function of distance, 100 m to 100 km, for particles having v_g = 2 and 4 cm/sec. (after Chamberlain, 1956).

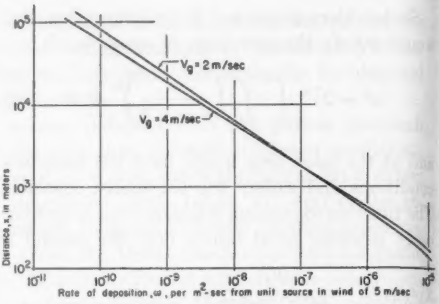

FIG. 3. Deposition rate, ω, directly downwind from ground level source as a function of distance, .15 to 100 km, for particles having v_g = 2 and 4 cm/sec. (after Chamberlain 1956).

an integration can be made over all sizes of raindrops. Using rain spectrum data given by Best (18), Chamberlain finds for *Lycopodium* spores of 16μ diameter that the attrition is 0.03%/sec. for a rainfall rate of 1 mm/hr.

Figures 2 and 3 summarize Chamberlain's results along the 'plume' axis.

c) Relative merits of the theories. Direct comparison of the results of the two theories is not readily achieved. In practice it has been found that Sutton's equations agree much more nearly with observations in several cases than do those of Schmidt. There is wide agreement that the statistical theory applies to atmospheric diffusion much more adequately than molecular diffusion theory does. In studying the applicability of Chamberlain's results to the problem at hand, namely, the dissemination of ragweed pollen, it is seen that a direct adaptation can be made.

Fortunately the *Lycopodium* spores studied by Gregory (16, 19, 20) and by Chamberlain are similar aerodynamically to *Ambrosia* pollen grains. The pollen grains have slightly greater diameter than the spores, but the terminal fall speeds are nearly the same. Crawford (21) gives 1.5 cm/sec. for the fall speed of *Ambrosia* pollen, and Gregory (19) indicates that *Lycopodium* spores fall at about 1.6 cm/sec. Thus it is reasonable to estimate that both of these particulates have v_g values near 2.0 cm/sec. at wind speeds near 5 m/sec., and that Chamberlain's curves for v_g = 2.0 cm/sec. and his results for *Lycopodium* spores apply also to *Ambrosia* pollen.

2) Ragweed Pollen Transport in Outdoor Air. It turns out when one presses for details, that relatively little is known with any degree of certainly about the exact process whereby ragweed pollen grains leave their respective anthers and become airborne. One view is that, when the anther ripens, it opens violently and ejects its pollen more or less explosively. Another is that the ripened anther opens more gradually exposing the pollen to the atmosphere more or less passively. Gusts and eddies then must provide all the requisite energy to shake the pollen grains free and bear them aloft. The latter mechanism must be regarded as much less efficient for placing pollen into a gently flowing air stream than the 'anther explosion' mechanism. When the air stream is more swift and hence also more turbulent, the release mechanism probably becomes less important. However, it is quite well established that most ragweed anthers open in the morning as soon as the dew has evaporated. This is a time of relatively gentle air motion usually, so that it may be quite important which mechanism actually operates. This point is under study by the botanists in the aeroallergen project at the University of Michigan.

Atmospheric turbulence operating upon the space and time distribution of pollen grains released directly to the air stream determines the pollen dissemination pattern in the early part of the pollination season. Thus one should expect the dissemination pattern to be quite complex (22). It is of interest both to the meteorologist and to the allergist to know the nature of these patterns in relation to more readily measured parameters.

a) Experimental studies of aerial distribution of pollen. Some experiments were conducted in 1955 at the University of Michigan for the purpose of defining the behavior of a cloud of pollen

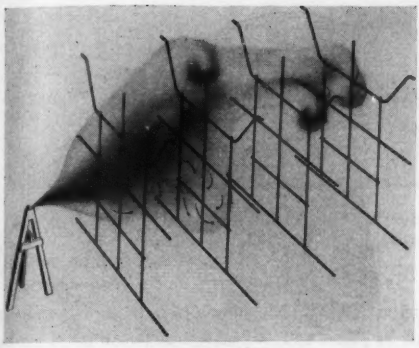

FIG. 4. Reconstruction of dispersal pattern of ragween pollen emitted from a point source, inferred from a three-dimensional array of impaction samples. Total emission time 30 sec. (Hewson and Dingle, 1956).

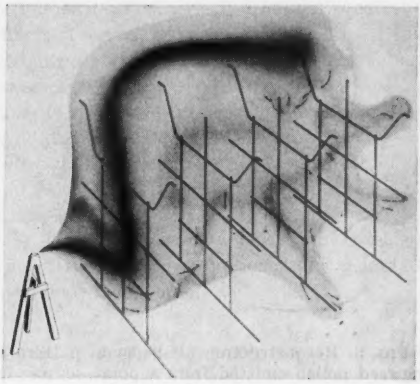

FIG. 5. Reconstruction of dispersal pattern of ragweed pollen emitted from a point source, inferred from a three-dimensional array of impaction samples. Puffwise emission over 90-sec. period (Hewson and Dingle, 1956).

released suddenly into the air stream (23). The results of these are shown graphically in figures 4, 5, and 6. By confining this experimentation to periods other than the natural pollination season for the *Ambrosias*, ragweed pollen could be used as a labeled particulate. The source in these experiments was a small dust atomizer, and a three-dimensional sampling array was used. Each of the four collapsible frames supported 19 impaction sampling stations, and at each of these stations were mounted one coated microscope slide facing upwind and one facing vertically upward. In the first two experiments (figs. 4 and 5), the array was oriented to be downwind from the source. In the third experiment, the objective was to bathe a small test room (fig. 6, low-roofed part of superstructure), with the pollen cloud using the sampling array as a means of monitoring the cloud. Partly for this reason, but principally because other roof obstructions prohibited it, the array was not wind-oriented in this experiment.

The air brush has been used to show gradation of pollen concentrations as they are inferred from the impaction patterns, and to help emphasize the three-dimensional character of the flow. The artist's dramatic inter- and extrapolations outside the sampling array help the effect but do not constitute data.

Wind data were also taken during these tests, but the scale of the array is small compared to that of the turbulence elements, and hence statistical theory cannot be applied properly to the observations. Detailed observations of the wind structure and of the distribution of pollen concentrations, taken in accord with a valid statistical design, appear to hold the most promise of illuminating the pertinent relationships. Further experimentation is contemplated, but obviously the question of scale is a difficult one.

b) *Problem of reflotation.* It appears almost certain that some pollen grains are picked up from their rest positions and refloated by the action of turbulent eddies. Thus even though a particular ragweed patch may have ceased pollinating, as long as heavy deposits of pollen lie on the leaves, such a patch will remain a source of airborne pollen. Furthermore, because of this process of reflotation, the sources of airborne pollen are identifiable with blooming ragweed only during the early part of the pollination season. As time progresses, any surface from which a resting pollen grain can be refloated becomes a potential source. How far this process may go is not known. It is subject to investigation with some difficulty by the use of 'marked' pollen, which may be rendered identifiable either by such a device as radioactive tracers or by the expedient of liberating pollen from a limited source 'out-of-season'. The latter can be done either mechanically as described above, or botanically by growing ragweed in the greenhouse and forcing it to bloom 'out-of-season' by controlling the photoperiod. Needless to say, the use of 'marked' pollen offers many possibilities for profitable research.

c) *Marked pollen in deposition experiments.*

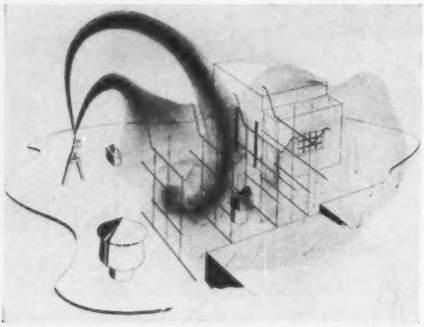

FIG. 6. Reconstruction of dispersal pattern of ragweed pollen emitted from a point source, inferred from a three-dimensional array of impaction samples. Puffwise emission over 110-sec. period (Hewson and Dingle, 1956).

It is appropriate to mention one use to which the greenhouse-forced-blooming method has been used at the University of Michigan. In this experiment, a number of hothouse-reared ragweed plants were brought to the blooming stage in late June 1956. These were then set out in a mown meadow and surrounded by upward-facing impaction samplers at distances of 10, 20, 40, 80 and 160 ft. The samplers were supported 2 ft. above the ground and were set on radial lines at 40° intervals. Pollen counts from this experiment have been totaled in figure 7. To construct this figure, the counts for each sampling period have been oriented according to the rules that the sampling ray nearest the total wind vector for the period must be directed along the $+x$ direction, and that the wind speed must average at least 5 mph. As a result, figure 7 presents a composite average picture, over a period of 31 hr, of the deposition from the pollen 'plume'.

The figures represent pollen deposition reduced to the average number of pollen grains caught in 6 hr. on 1 cm² of coated slide surface. The symmetry of the 0.5 line, which forms the outer limit of shaded area, is remarkable, especially when viewed in contrast to the asymmetry of the progressively denser areas near the source. The occurrence of the high counts far to the left of the average wind direction serves to emphasize the fact that the deposition patterns are produced by two factors, the wind, and pollen availability. The interaction of these two is probably of great importance near the source where extremely high concentrations of pollen are encountered. Very small-scale, short-lived gusts or eddies

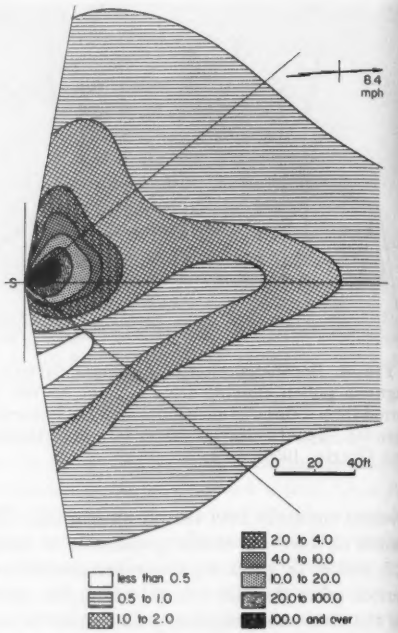

FIG. 7. Composite of ragweed pollen deposition at wind speeds greater than 5 mph as constructed from impaction samples taken 2 ft. above the ground. Source, S, was a 4-ft-square bed of hothouse-forced *Ambrosia elatior* set out in a mown meadow. Deposition values are grains per cm² in 6 hr.

occurring at the time of maximum pollen release rate may contribute heavily to the deposition pattern without contributing substantially to the average wind vector. On the other hand, the more distant deposition pattern depends most heavily upon the average wind vector, and only slightly upon the wind details at the time of pollen release. Hence it would appear that such equations as that of Chamberlain (*equation 22*, above) are probably best applied to the more distant portions of the 'plume' to which generalizations such as the average wind speed can in fact apply. Reflotation of pollen grains is an additional factor which would tend to respond to the average wind vector and to improve the symmetry of the deposition patterns with increasing distance. It is not clear at this juncture whether the reflotation effect can be isolated to improve the applicability of Chamberlain's equation, but additional experimentation designed to evaluate reflotation directly is contemplated.

3) Ragweed Pollen Penetration Into Enclosed

FIG. 8. Field test house for ragweed pollen penetration studies showing outside instrumentation. (Hewson and Dingle, 1956).

Spaces. From the allergists' viewpoint, pollen refuge is an important concept. The study of the penetration of ragweed pollen into buildings of various types by virtue of natural ventilation is pertinent both to this concept and to problems of controlled ventilation and air-conditioning from the engineering point of view.

a) Experimental work. Studies of the penetration of ragweed pollen into a standard construction field-office structure (fig. 8) have shown that the natural ventilation of the building and the resulting pollen penetration depend both upon the wind speed and the wind direction (23). Although gustiness is probably responsible for most of the observed effect, the wind speed appears to be as good as any existing gustiness index for explaining observed pollen penetration rates.

One general observation that is significant for the interpretation of pollen transport and penetration situations is that there are ordinarily thresholds of wind speed above which certain specific effects are observed. For example, pollen penetration into the field test house was insignificant until the wind exceeded 1 mph. Above that wind speed, penetration rates increased with increasing wind, but not uniformly. Dingle (24) found that a wind of about 15 mph was needed to elevate large numbers of pollen grains to a sampler stationed on the roof of a building, about 60 ft. above the ground. In studies of the movement of sand by the wind, Bagnold (25) reports that a wind of 11 mph, measured 10 cm above the sand surface, starts movement of sand grains, and Landsberg and Riley (26) state that a wind of 8 mph, measured 5 cm above the sand surface, produced movement of the sand grains. The thresholds having to do with the lifting and moving of particles at and near the ground may be primarily associated with the breakdown of the surface boundary layer of air rather than with the aerodynamic properties of the particles concerned. As to the penetration effects observed, it appears that increased wind turbulence, with the associated locally induced pressure variations, serves to 'activate' crackage areas of a building that remain effectively closed at lower wind speeds. Thus the ragweed pollen penetration rate is augmented by virtue of the two factors, and its relation to wind speed is not a simple curve.

b) Theoretical treatment. Theoretical consideration of the pollen penetration problem leads to the differential equation

$$\frac{dx}{dt} = k(m - x) - \frac{wx}{h} \qquad (25)$$

which expresses the rate of change of the pollen concentration in an enclosed space (dx/dt) in terms of the ventilation rate, k, the pollen concentrations in the space, x, and in the outdoor air, m, the fall speed of the pollen grains in still air, w, and the vertical height of the room, h. In view of the fact that the value of m, the outdoor pollen concentration, is always changing, the dynamic nature of m should enter into the solution of this equation. For cases in which m varies sinusoidally with time, $m(t)$ may be written

$$m(t) = C + B \sin \omega t. \qquad (26)$$

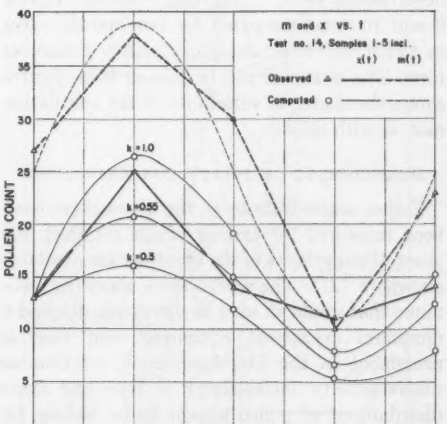

FIG. 9. Time variation of ragweed pollen concentrations inside (x) and outside (m) a test house as shown by a series of 5-min. samples. and as computed from *equation 27* for several values of the ventilation rate, k (Hewson and Dingle, 1956).

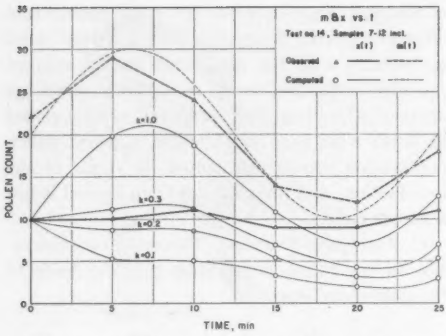

FIG. 10. The time variation of ragweed pollen concentrations inside (x) and outside (m) a test house as shown by a series of 5-min. samples, and as computed from *equation 27* for several values of the ventilation rate, k (Hewson and Dingle, 1956).

The solution for $x(t)$ is then

$$x(t) = x_0 e^{-At} + \frac{kC}{A}(1 - e^{-At}) + \frac{k\omega B}{A^2 + \omega^2}(e^{-At} - \cos \omega t) + \frac{kAB}{A^2 + \omega^2} \sin \omega t \qquad (27)$$

where x_0 is the value of x at time $t = 0$, $A = (k + w/h)$, and B, C and ω are derived from observations of m. Using this equation, figures 9 and 10 were computed for two specific cases in which $m(t)$ took an approximately sinusoidal form. The course of $x(t)$ in each of these figures shows the degree of variability of the ventilation rate, k, with time.

MEASUREMENT OF POLLEN CONCENTRATIONS

Pollen concentrations in the atmosphere have been measured by various means for over 100 years. Good reviews of the literature are available elsewhere (27). The search for a sampling technique that would at once be direct and demand a minimum of special equipment and time is prominent in the literature. Such concerns as inhomogeneity (anisotropy) of time and space distributions of pollen appear to be lacking for the most part until recently (22, 23, 28), thus much misunderstanding of the meaning of and uses for pollen counts has been propagated. It is these problems to which a few comments may be appropriately addressed.

1) Atmospheric Distribution of Pollen. It has been pointed out above that by virtue of the superposition of the complex of turbulent elements of wind upon the irregular time and areal distributions of pollen release, one must anticipate that the number of pollen grains per unit volume of air will vary rapidly and widely from place to place and time to time. This fact obviously requires consideration in establishing a sampling procedure.

It is clear that the concentrations cannot be determined unless, in the sampling procedure, the volume of air sampled is measured. Depending upon the wind speed, the turbulence pattern, and the time over which a sample is collected, the shape of the air sample will vary. That is to say that when very little wind is blowing, the sampler will draw air from an adjacent space of relatively compact shape (fig. 11a), whereas when the wind is stronger, the air sample will be drawn from a long thin space having branches or tributaries contributed by turbulent eddies (fig. 11b and c). This latter is probably the most usual condition in outdoor air. That this is true introduces an additional consideration which again is hard to quantify, namely, the role that turbulent eddies play in impacting pollen grains upon or removing them from a filter surface independently of the volume of air drawn through the filter. The *Millipore* filter probably retains quite well the pollen grains that impact upon it, but it is not known at the present time how much of the collected sample is contributed by the centrifugal action of eddies near the filter head.

The ideal sampler according to Magill (29) would operate isokinetically. That is to say, it would operate in such a way that the natural flow streamlines would not be deviated in any way. There exists no apparatus that can accomplish this ideal for the simple reason that just the presence of the sampler in the air stream requires some deviation of the streamlines, even for an air foil shape. The turbulent condition of

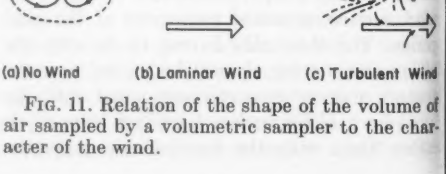

FIG. 11. Relation of the shape of the volume of air sampled by a volumetric sampler to the character of the wind.

the natural flow is an additional factor that makes this ideal unattainable in practice, because such a sampler must always face into the wind and must filter the air at a rate equal to the wind speed at all times. The isokinetic criterion is therefore best regarded as a design guide for use in devising the best possible samplers. The volumetric samplers currently in use at the University of Michigan (fig. 11) do not operate isokinetically and, as a result, probably indicate concentrations of contaminant somewhat below those actually existing. There are, however, a number of other points at which error enters the sample evaluation procedure, and it is believed that the error due to non-isokinetic sampling is quite small compared to the natural variability of concentrations and to the cumulative error introduced in handling, counting etc.

2) Meaning of 'Pollen Counts'. Thus the meaning of a pollen sample changes drastically with the conditions of air movement under which it is collected, and it is very difficult to establish a good model that would represent the spacial distribution of pollen grains in the air at any instant. Because of these factors, it must be recognized that an isolated pollen count has complete validity only for the place and time of its collection. It does not necessarily characterize the pollen exposure of any particular hay fever patient, and by long odds it probably differs greatly from his exposure. This should serve in part to explain the disparity between the traditional 'pollen counts' and the clinical symptoms displayed by individual patients.

Inasmuch as the traditional 'pollen count' is a 24-hr sample accumulated upon a 'gravity slide', one should understand that it has no validity whatever as a representation of the number of pollen grains per unit volume of air. Further, because it is usually collected at a local hospital or U. S. Weather Bureau observing station, it cannot, except in the most tenuous way, characterize the general level of air pollution by pollen grains. Clearly if there is to be an official, published pollen count for a city, it ought more properly to be an average of numerous volumetric samples taken so as to characterize the bulk of human environments in the city. As an example of urban micro-distribution of dust, figure 12 is reproduced from Löbner (30). Here are shown gradations of dust at a street intersection in Leipzig. At which point should one sample to determine a representative 'pollen count'?

3) Function of Pollen Counts. Although the above commentary appears discouraging as regards the broad use of pollen counts, it is perfectly clear that, for purposes of research and treatment, some monitoring of the atmosphere for pollen concentrations is important. The difficulties cited above introduce problems of interpretation into the question of monitoring however. Because these difficulties are primarily associated with the variability of states and patterns of motion of the atmosphere, it is apparent that they can be resolved only by taking the atmospheric variables into account. When this is done, upon the basis of relationships derived from adequate research, almost any kind of sampling technique that may be used in conjunction with suitable atmospheric measurements will be subject to realistic interpretation. At the present time, the basic requirement appears to be that of establishing relationships between pollen distribution and suitable weather parameters. These are under study.

Again, inasmuch as the pollen counts are useful, they would probably be more so if they were predictable. In this event preventive therapy could be used for serious chronic allergic patients. To the extent that the phenology of the source plant is known, the release of allergenic material will be predictable from accumulated standard-

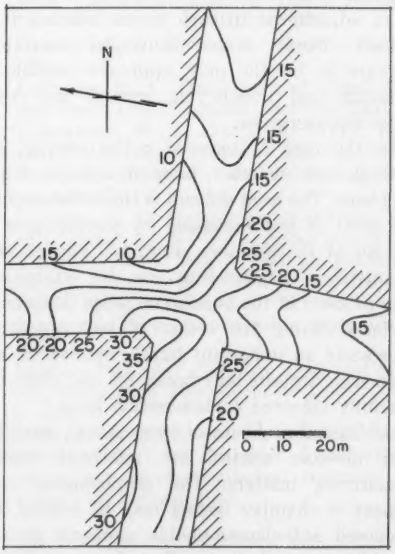

FIG. 12. Dust distribution at a street crossing in Leipzig. Dust content figures give numbers of particles per cm³ (after Löbner, 1935).

ized weather observations. Knowledge of the relation of distribution patterns of the allergens to atmospheric conditions will then render these and hence the general level of pollen concentration predictable.

Note now, that these predicted levels of pollen concentration, predicated upon knowledge of the state of the atmosphere and the production patterns of the source plants, will actually have greater validity than any particular sample or group of samples can have for describing the general level of concentration of allergenic substance in the air. The clinical usefulness of such predictions should be immediately apparent. Thus probably the most important meteorological objective of the aeroallergen research program, aside from control of the source, is to establish those relationships that will render the level of contamination predictable.

CONTROLLED EXPERIMENTATION

There remains an aspect of the total problem that has not been discussed. This is the patient. Fortunately for the patient in most cases, but unfortunate as regards progress in research, the human organism has so many built-in checks and balances that its responses become obscured. In addition, as regards allergies especially, there are a multitude of stimuli to which the organism responds, and the intensity of response or of adjustment to each varies between individuals. Study under controlled conditions appears to be the only approach capable of isolating and quantifying stimuli and their respective responses.

In the case of ragweed pollen allergy, the clinical test chamber presents special design problems. The most difficult is that of controlling the level of contamination by aeroallergens in the air of the chamber accurately enough that quantitative relationships can be established. The problem of the buildup of pollen deposits on surfaces during the course of test operations represents an important hurdle because at any time such deposits may break off and disturb a carefully adjusted contamination level.

Additional problems of temperature, humidity and pressure control are relatively routine engineering matters. The psychological component of chamber testing may be critical, but proposed anti-claustrophobia measures such as the liberal use of glass walls and communication facilities are available. The free use of glass introduces the problem of radiational balances which may be important in relation to comfort, and which are not so much present in opaque-walled chambers.

Two serious difficulties remain, however, in the specification of test conditions and responses. The first is the absence of a satisfactory quantitative measure of the antigenicity (or allergenic potential) of pollen grains. There is subjective evidence that suggest differences in this characteristic, and independent knowledge of plant chemistry indicates that such differences should be expected. They are believed to be related to the life-weather-history of the plant and to soil nutrient differences in addition to species, subspecies and variety differences. But the critical substance that stimulates the allergic reaction remains a natural secret the key to which has not yet been identified.

The second difficulty is the qualitative and subjective criteria that we depend upon to document allergic reactions. The processes of human body chemistry that operate between the impaction of the pollen grain upon the nasal mucosa and the sniffles and sneezes that follow appear to remain quite obscure.

These are problems of immunochemistry, a field that contains no simple problems. Even with test chambers, completely satisfactory results can be obtained only to the extent that quantitative statements can be made concerning exposure and reaction. Currently it looks as if the best attack would be an immunochemical one.

SUMMARY AND CONCLUSIONS

It has been shown that meteorological considerations figure prominently in the total problem of ragweed hay fever. They are important in the states of plant growth from germination to blooming, and their significance in this period is best established by means of phenological research. They enter the field prominently in the pollen transport, bloom to patient, process. In this process atmospheric turbulence is predominant. Despite its complexity, theoretical development in the turbulence field is continuous and some useful applications to problems of pollen dispersal are being made.

Experimental work designed to yield relationships between meteorological parameters and the spacial distribution patterns of aeroallergens is largely within the meteorological province. The

penetration of aeroallergens into buildings also involves meteorological considerations.

Atmospheric sampling to determine contamination levels and the production of controlled contamination levels in test chambers have become primarily problems of meteorological engineering.

Finally, the principal objective of aeroallergen research remains that of reducing the prevalence and magnitude of the reaction that is called hay fever disease. This may be accomplished either by reducing the prevalence of aeroallergens through suitable control measures or by reducing patient reactions through preventive treatment born of a more complete knowledge than now exists of the immunochemistry of hay fever and made feasible by reasonably good predictions of aeroallergen quality and quantity. The problems of botany, of meteorology, of immunochemistry and of clinical medicine that arise in the attainment of this broad objective are so interdependent and so complex within themselves that an effective attack upon them can be launched only by a sustained cooperative effort. The research project on Air Pollution by Aeroallergens at the University of Michigan is this kind of an endeavor.

The June, 1956, experiment (fig. 7) was instrumented and supervised by Mr. G. C. Gill of the Meteorological Laboratory with the cooperation of Professor W. H. Wagner, Jr., of the Department of Botany, who also assumed responsibility for supplying the hothouse-reared ragweed in a blooming stage. The field test house (fig. 8) used for the penetration experiment was delivered, placed and finally removed gratis through the kind offices of Mr. John Armstrong, president of the construction firm of Darin and Armstrong, Inc., Detroit. Enthusiastic support and continuing consultation on the experimental program have been supplied generously by Professor E. W. Hewson, Department of Civil Engineering. Without the special cooperation and conscientious effort of the field crews including Mr. A. W. Wagner, technician, and Messrs. J. Asuncion, J. A. Ismaili, D. A. Lundy, K. C. Quan, and P. Rangaswamy, the collection and reduction of the large masses of data required (figs. 4, 5, 6, 7, 9, and 10) would have been impossible.

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AIR POLLUTION BY RAGWEED: MEDICAL ASPECTS[1]

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Hay fever and asthma occur in approximately three to four million people in the United States, and these two diseases rank third among the chronic diseases. Asthma occurs in approximately 30–45% of patients with hay fever.

In order to better understand these conditions, this study was carried out on patients whose symptoms were due only to ragweed sensitivity or who had a definite seasonal exacerbation during that period. Preliminary studies on clinic and private patients revealed that many variables could not be controlled, one in particular being the wide range of movement and change of geographic location during a season. Therefore, studies were carried out on volunteers at the state prison in southern Michigan. A complete allergic evaluation was made and 11 subjects were selected as the most suitable. Of these, 4 had only hay fever, and the other 7 had both ragweed hay fever and asthma. None were receiving hyposensitization and a few took an occasional antihistamine. Three subjects (1 hay fever and 2 asthma) were confined to a small hospital ward for 3 weeks of the season. Windows were kept open and continuous, quantitative pollen counts were made in 2 of the subjects' rooms and in the connecting hall. Continuous quantitative pollen counts were also done on a 100-foot tower approximately one mile from the prison; these were done at 4-, 50-, and 100-foot levels along with the collection of meteorological data. The quantitative pollen counts were made by means of a multiple head unit, each head containing a molecular filter membrane (1). Air was drawn through the filters by a vacuum pump, a limiting flow orifice in the system insuring an accurate control of the flow rate. An electric timer and a solenoid switch permitted changing from one head to another every 4 hours, counts being made successively throughout each 24-hour period. A standard 24-hour gravity slide pollen count (2) was also collected on the prison hospital roof each day.

The 3 men in the prison ward were examined twice daily; the remaining 8 followed their usual prison routine and were examined once daily; each man was examined at approximately the same time each day. Pertinent physical findings were checked and a quantitative blood eosinophil count was done. Salivary smears and pneumotachographic records were made but will not be discussed in this paper. Maximum expiratory flow-rate curves, timed vital capacities and MBC's and A.V.I.'s (air velocity index) were done on each man daily.

All subjects also kept a daily record of their hay fever and asthma symptoms; these symptoms were rated for each 4-hour period of the day, the periods corresponding to the quantitative pollen count time periods. These cards were collected and checked each day when the patient was examined.

The M.E.F.R. test is done by having the patient take a maximum inspiration, then rapidly and forcibly exhaling maximally; this expiration is recorded on a rapidly moving drum and the rate of flow is measured between certain fixed reference points. The normal value is between 400–500 l/min. (3).

The vital-capacity test was used because it is simple and is a test usually done by clinicians. The timed V.C. is more meaningful because it establishes limits in relationship to time. Our values were compared to the standards accepted for body surface area.

The MBC is an aid in obstructive disease of the pulmonary system because it also takes into consideration the time factor. The test is run by having a patient breathe in and out as rapidly and forcibly as possible for a known period of time while a kymograph records his respirations. However, it is a more difficult test for the patient to perform and requires the utmost cooperation. Thus, in severe asthma it may be unreliable. The normal values were calculated from Baldwin's formula (4).

Quantitative blood eosinophil counts were done because the eosinophil count is known to rise in some allergic conditions, and counts above 400 eosinophils per cubic millimeter were con-

[1] This investigation was supported by Research Grant E-1379 (C) from the National Institute of Allergy and Infectious Diseases, Public Health Service: Publication No. 3.

sidered to be significantly elevated. Counts on patients in the hospital ward were fasting while the others were not; diurnal variations were not specifically investigated with multiple daily counts (5).

All of the patients had definite hay fever; 6 had spontaneous asthma, but only 1 had moderately severe asthma. In the 6 patients having definite asthma, using the standard calculated values, we found that the following number of pulmonary ventilatory tests were abnormal; these were divided into 10-day periods with the second period corresponding with the season's pollen peak:

Asthma	Aug. 13–26	Aug. 27–Sept. 7	Sept. 8–21
Abnormal Pulmonary Ventilatory	163	131	136
Elevated quantitative eosinophil counts	18	24	24
Total	181	155	160
Hay fever	40	28	23

Thus, the number of abnormal tests was not elevated during the pollen peak as we had anticipated. These abnormal tests in the 3 hay fever patients consisted of abnormal pulmonary ventilatory function studies except for only 2 abnormally elevated quantitative eosinophil counts. In all 3 hay fever patients the 1′V.C. was abnormal 66 times, and in 2 of these patients the M.E.F.R. was abnormal 19 times. This may indicate the detection of minimal asthma which was not clinically apparent; others (6–8) have also noted this finding. The 1′V.C. was the pulmonary ventilatory test which was most frequently abnormal in the asthmatic patients, 229 times. However, these two tests (M.E.F.R. and 1′V.C.) were not abnormal in the hay fever or asthmatic patients with greater frequency during the peak of the pollen season.

Although we could not rely on a given total V.C. level, we noted that our patients fit into Feinberg's classification (9): that is, a total V.C. of 73% with tightness in the chest, 60% in a moderate attack, and 41% of the expected value in severe asthma. Three of our patients having the most days of moderate asthma had total V.C.'s in the range of 61, 62, and 67%; those with minimal asthma had their lowest total V.C. in the range of 73, 77, and 78%. One asthmatic patient's total V.C. never was lower than 82%. One hay fever patient with no subjective sensations of asthma (although he did have objective evidence of minimal wheezing) had a total V.C. in the 77% range.

An interesting observation is that the one-second timed vital capacity appears to correlate better with the patient's own subjective wheezing rating than with our objective wheezing evaluation. In addition, the one-second timed vital capacity appeared to correlate well with the size of the nasal airway, nasal color and discharge, conjunctival color and discharge, and pulse rate—all being objective data. Subjectively this same correlation seemed to appear with the choking sensation, and out-of-breath at rest and while walking. No correlation was noted with the respiratory rate, tightness in the chest or cough. All of these data, however, are on a limited number of patients.

In general as the MBC increases so does the M.E.F.R. as would be expected. The 1′V.C. appeared to correlate better with the M.E.F.R. than did the total V.C. When the total V.C. is compared to the 1′V.C., it is seen that the lower values occurred in the asthmatic patients while the hay fever patients had the higher test results (table 1). The AVI was not helpful in this study. All of these data will be subjected to statistical analysis.

It should be noted that the total asthmatic subjective symptoms tapered off gradually at the end of the season while the hay fever symptoms stayed higher throughout most of the usual season (fig. 1). The average hay fever symptom severity count for the asthmatic patients was higher than in those with hay fever alone, or, the patients with more severe hay fever were more likely to have asthma. Asthma patients had 17% more hay fever by subjective rating than did patients only experiencing hay fever. Objective asthma symptoms started after objective hay fever symptoms in 3 patients, on the same day in 1 patient, and actually they started 1 week before in another patient.

TABLE 1. TOTAL VITAL CAPACITY

	2–2.9 L	3–3.9 L	4–4.9 L	5–5.9 L
1′ V.C.				
1.9 L	1 A			
2.2 L	20 A	94 A		
3.6 L		71 A	49 A	1 A
		5 HF	63 HF	20 HF
4.0 L			11 A	
			3 HF	18 HF

A = Asthma. HF = Hayfever.

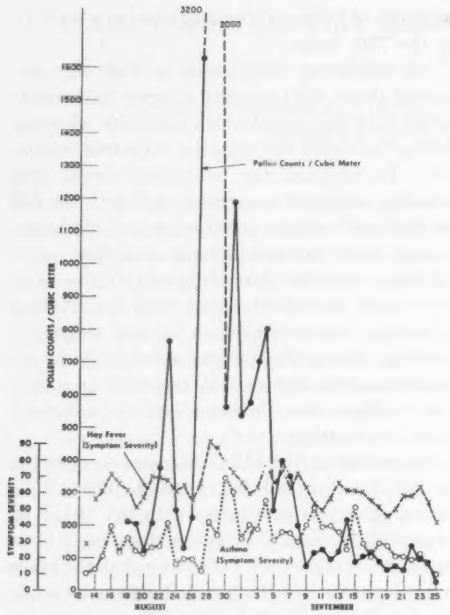

FIG 1. Quantitative volumetric pollen counts and hay fever and asthma severity.

There was a fair agreement between objective and subjective asthma and hay fever ratings with the pollen counts when the total pollen season was considered. Actually, subjective ratings appeared to follow the general pollen season the best. There were many variations among individual patients, however. Also, again considering the total patient subjective symptomatology (fig. 1), there is a simultaneous decrease in hay fever severity and an increase in asthmatic severity on September 10.

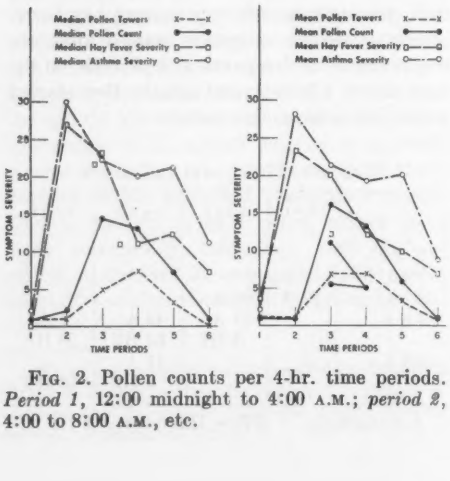

FIG. 2. Pollen counts per 4-hr. time periods. *Period 1*, 12:00 midnight to 4:00 A.M.; *period 2*, 4:00 to 8:00 A.M., etc.

The pollen peak for the season occurred on August 28, and the highest hay fever subjective symptomatology rating occurred on that same day while the highest asthma subjective symptomatology occurred 48 hours later. A significant pollen rise occurred on September 13 with no increase in hay fever symptomatology and a 24- to 48-hour lag in the increase in asthma subjective symptomatology. However, isolated pollen rises occurred on August 23 and September 3 and 4 with only a slight rise in hay fever and/or asthma symptomatology at the same time and no delayed increase in the asthmatic patients.

The specific daily pollen count patterns are of interest (fig. 2). The mean and median pollen counts showed their greatest increase and rose to the daily pollen peak in the time period from 8:00 A.M. to 12:00 noon, (*periods 2–3*) while the mean and median subjective symptom increase for both asthma and hay fever occurred prior to this, namely between 4:00 and 8:00 A.M. (time period *1–2*). The rise in pollen counts as reflected by the 100-foot tower readings also reflected this same lag and it was even further delayed to the period from 12:00 noon to 4:00 P.M. This apparent discrepancy has been noted by others (10); a diurnal variation in symptoms with an increase upon arising in the morning (6:00 A.M. at the prison) may partially explain these findings. Other experiments (ragweed plants were made to mature earlier in the season and the pollen dispersal was studied in this 'non-seasonal' interval) revealed that pollen was collected in highest concentration between 5:00–8:00 A.M. on slides exposed at two-foot level above the ground. Thus, pollen emission may occur early in the morning and later; air turbulence carries this pollen to the higher elevations.

SUMMARY

Using the experimental approach described in a study on ragweed pollinosis, the following observations were noted: *1*) in general, the patients' subjective symptoms seemed to follow the general seasonal pollen pattern better than did their objective findings, although there were many exceptions. *2*) In asthmatic patients the 1'V.C. was abnormal more times than any of the other pulmonary ventilatory tests. In the hay fever patients the 1'V.C. and the M.E.F.R. were abnormal occasionally. The quantitative blood eosinophil counts and pulmonary ventilatory

function tests were not abnormal, with a greater frequency during the peak of the pollen season. 3) The rise in pollen counts in the morning followed rather than preceded the daily increase in the subjective symptoms for both hay fever and asthma. Possible explanations for this were cited.

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PRESENT KNOWLEDGE ON CORRELATIONS BETWEEN WEATHER CHANGES, SFERICS AND AIR ELECTRIC SPACE CHARGES, AND HUMAN HEALTH AND BEHAVIOR[1]

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In the 9th century A. D. the law of the Frisians (lex Frisionum) imposed a higher weregild or tax for an inflicted wound if a weather-sensitive scar remained (1). This is the oldest known record of the very many complaints about weather-sensitive scars, complaints which are quite similar to those of rheumatics. On the other hand, the Föhn or Chinook wind has been blamed for centuries for its adverse effects on man. The scar and the Föhn complex are the classical enigmas of statistical bioclimatology. No factual evidence has disproved these observations, or has successfully explained the effects. Around these center complexes we find a large number of suspected weather-illness correlations of all kinds. As time passed, complexes strayed into and out of this field, some being solved, abandoned, recognized as pseudo-problems etc.

The relationship investigated most often has been any possible correlation between sudden illnesses and air pressure variations of all frequencies. This correlation has always proved to be poor (2). The pressure variations from staircases and elevators, as well as from wind, generally exceed the meteorological ones, and should be equally effective on man if small pressure variations are effective at all. Of course, this problem has nothing to do with the effects of large pressure changes, a field of aviation physiology. Similarly, temperature, sunshine and wind speed can hardly explain the scar or Föhn complex since none of them penetrates modern houses. A host of similar observations on other illnesses and physiological and mental reactions in man have been reported. These correlations are part of statistical bioclimatology which investigates, on a strictly statistical basis, changes in man's actions, reactions or illnesses with respect to time and location. This is a field of science which is alternatingly praised or held in contempt. By all standards of definition, malaria, altitude sickness, skin cancer, summer diarrhea of infants, hay fever and many epidemic diseases once belonged to this field. These illnesses now have 'advanced' to qualitative or quantitative bioclimatology, or interest in their climatological or weather relationship has ceased. On the other hand, many claims of statistical significance did not withstand critical tests, or are still unconfirmed by other authors or by physical experiment. The unconfirmed group contains correlations between solar activity and human deaths (3). This problem is, of course, related to the search for any measurable effect on tropospheric factors by variations of solar activity; this search has begun. To the abandoned group belong the Takata radiation

[1] Contribution No. 27 of the Dept. of Meteorology and Climatology, Univ. of Washington.

theory (4), the Gehrke dust theory (5), the 'Aran' hypothesis of M. Curry (6), the claimed relationships between menstruation and the moon and between births and tides, and many assertions based on an oversimplified, meteorological 'air mass' hypothesis.

Beyond the still debatable claims is a host of pseudoscientific stories of every caliber; they show a curious trend toward attempting to discover mysterious rays, from earth rays over death rays to Gurwitsch rays and solar neutrinos. Their making press headlines is as comforting as headlines about flying saucers or the sea serpent of our grandfathers. These headlines usually indicate a lull in politics.

In view of all this confusion, is it worth our while to evaluate the present situation in this field? I think it is. We have, however, to be quite aware of what statistical evidence is.

When the ship's doctor of the sailing ship *Hispaniola* inspected the harbor of Treasure Island he groaned, "The nasty stench of the place turned me sick; if ever man smelt fever and dysentery, it was in that abominable anchorage." Thus, he correlated the foul odor of tropical marshes with malaria. As we know now, this type of argument helped in discovering the causes of malaria, and can be called a productive kind of statistical bioclimatology. A positive correlation also exists between the number of people drowned while bathing and acute polio cases. This is an unproductive approach, since both factors have to do with the summer, and their relation belongs to the rhythm research. The difficulty lies in the fact that nearly all biological factors in man have a yearly and a daily period; so do nearly all meteorological factors. Any correlation between the two, then, shows that both changes take place on a revolving and rotating earth.

The statistical approach is frequently the first step toward learning the truth. In statistical bioclimatology the causative environmental agent is generally unknown, frequently not even suspected. All we know are the coincidences, the frequency patterns, the correlations etc. This approach does not always guarantee a tangible result of a research. Time prevents my describing the interesting history of our fied. Major work has been done in this country by W. F. Petersen (7), E. Huntington (8), and C. A. Mills (9). The first to request day-by-day relations of illness and weather (to study the common cold) was F. Sargent. The pertinent text book is that of B. de Rudder (1) which, strangely enough, seems still to await a translation and general texts on bioclimatology (10–13). In the following lines, I shall concentrate on the post-war period. Grants from the USAF Office of Scientific Research and USAF Arctic Aeromedical Laboratory have enabled me to discuss matters with workers in the field during meetings in Paris and Karlsruhe, and visits at the bioclimatic observatories at Hamburg, Frankfurt and Königstein, Tübingen, Bad Tölz and Garmisch-Farchant. Of the many working methods, a few shall now be discussed.

a) **Rhythm Method**. This includes the seasonal peaks of illnesses, and physiological and psychological deviations, as well as daily periods of similar kind. The list of illnesses with seasonal peaks is still staggering. The obvious reason is frequently thermal; conditions are too hot or cold for man, or they are more or less advantageous for microorganisms and carriers such as insects. Not all seasonal peaks, however, can be explained this way. Certain seasonal skin troubles are obviously hygric in origin. The summer brings excess sweating. The continental winter lowers air humidity indoors to far less than 10%. This, in turn, dries the stratum corneum to the point of brittleness and causes many small skin breaks. This effect is more pronounced in, for example, North Dakota than in Arizona.

b) **The Geographic Method** compares maps of illnesses, etc., with maps of climate or other environment factors. The maps of frequency of age-corrected cancer and heart deaths in North America are surprisingly similar (14). A map of the frequency of cloudy days has a certain similarity to these two death patterns, but so have maps of air humidity, precipitation, the per capita income, industrialization etc. Unfortunately, a higher correlation coefficient does not always belong to the more 'correct' theory, and is therefore not a safe tool with which to differentiate between the possible explanations of the riddle of cancer geography.

The geographical and, possibly, climatic distribution of the onset of sexual development has frequently been discussed. A possible latitudinal effect on the age of menarche, however, competes with the possible effects of variable health, nutrition, sanitation, education, etc., and, more important, the average age of onset has shifted to the younger years within the last decades.

c) **Weather Method.** As explained above, this approach is the oldest known and is still the most interesting one today. Every advance in meteorology from the invention of the barom-

TABLE 1

Meteorotropic Situation	Meteorological Characteristics	Weather Types	Coincident Human Events
1) Free Föhn	Meridional situation of tropical type, cyclogenesis to the west, subtropic air in all levels to the south	Daubert IIa; Ungeheuer 3; Kuhnke I, 1, a	Increase of: deaths (Hamburg, Tübingen), circulatory disorders, epileptic fits, other mental troubles, industrial accidents, auto accidents, appendicitis, kidney colic, headaches, thrombosis, C-17 ketosteroids high
2) Fronts	West weather, central low, fronts and occlusions, stable and unstable ascending, coincides with sferics maximum	Daubert III; Ungeheuer 4, 5; Kuhnke II, 1	Angina pectoris, sum of all new illnesses in Tübingen, deaths in Munich, emboly, C-17 ketosteroids low
3) True Föhn *and* fronts in Munich		Ungeheuer 3, 4, 5	Increase of reaction time, stomach perforations, industrial accidents, number of doctor's calls
4) True Föhn only		Ungeheuer 3	Pathological increase of inner eye pressure
5) High sferics count	Frequent at fronts but also before fronts	Reiter	Auto accidents, pain of amputees, reaction time, birth rate, death rate, industrial accidents

eter to the discovery of advection of vorticity (15) has been used to explain sudden increases in the rate of deaths, illnesses, accidents and certain physiological observations. Winds, Föhn, fronts, air masses, descending air and other meteorological terms have been used to correlate weather with human factors. Few of the older publications are convincing since they lack statistical significance and since they are based on a rather oversimplified meteorological analysis, especially of the fronts. These fronts differ from each other and from the beginner's textbook case as much as the same illness varies in as many patients.

One should not confuse the search for the unknown weather factor with the influences of known factors. These are mainly thermal. If strong cooling in a cold front coincides with a meteorotropic or an unknown weather effect in man, statistical evidence of the latter becomes difficult to evaluate. Considerable effort went into a new type of weather analysis done at the bioclimatic observatories in Hamburg, Frankfurt and Königstein, Bad Tölz, Tübingen and Freiburg in Germany. Table 1 shows a composite of this recent work. A critical evaluation of some of the statistical methods used in these studies is given by Jessel (16). By necessity, the time unit to define a weather type is the day from midnight to midnight. Each day is generally given a code number such as one of six numbers, shown in table 2. Of these weather phases, half are generally caused by air mass changes and show bad weather with precipitation, strong winds, sudden temperature changes etc. The others represent the 'radiation' weather, with little advective changes near the surface.

Certain illnesses (table 1) are more frequent during the 'bad' days, with frontal passages (17–20). According to older literature (1) pain of scars and from arthritis also occur during weather of the frontal type. These findings are hard to evaluate for unknown factors in view of the obviously possible thermal and hygric effects from changes of temperature, wind, precipitation, air humidity etc. The number of sudden illnesses, during frontal weather, in people protected from thermal weather effects cannot, however, easily be neglected. Auto accidents in Hamburg are not higher during frontal days (21).

The most surprising feature is the high frequency of industrial accidents and deaths from all causes in southwest Germany (17), deaths in Hamburg (18), automobile accidents in Hamburg (18), circulatory disorders (18), epileptic fits and other mental troubles (18) on days with no serious change of any geophysical surface element. The weather pattern (17, 18) on these days is characterized by a strong flow of subtropic air to the North reaching roughly to the station concerned; the tropopause is higher, the

TABLE 2. WEATHER PHASES
(*After Ungeheuer (23) upper Bavaria*)

1)	Fine weather, early haze, moderate visibility
2)	Extreme, fine weather, early naze, good visibility
3)	Föhn, extreme visibility, dry, warm, high clouds, lenticularis
4)	Beginning change, poor visibility, cooling, convection, precipitation begins, pressure low, maximum of sferics
5)	Full change, fronts, poor visibility, cool, convection, precipitation, sferics still frequent
6)	Beginning of postfrontal clearing, better visibility, cool, some showers, pressure high again, sferics decreasing

upper troposphere too warm and the lower stratosphere too cold. The 500- and 225-millibar maps show a high anticyclone to the SW or S and the jet stream to the N of the station; generally, cyclogenesis is present to the SW or W. Locally, air is subsiding, and might be warm in summer. This surface situation is, of course, similar to the alpine Föhn and might be termed a 'free' Föhn. During a 'free' Föhn, the C-17 ketosteroid level is irregular and high in patients completely at rest while hospitalized for broken legs or similar injuries. The ketosteroid level is low when cold air fills the lower and middle troposphere (22).

This 'free' Föhn, then, and the Föhn of the Alps are the center of the great enigma. The 'free' Föhn situation may be followed by 'bad' weather, in the well known pattern of a frontal passage, or the weather may stay 'fine'. One such example of free Föhn is the 3 December 1952 in Hamburg (18), when the death rate increased well above the 2σ level, σ being the standard error, and also coincided with a high rate of circulatory and mental trouble. These patients neither 'foresaw' nor felt any bad weather in the usual sense.

In upper Bavaria (23), the true Föhn takes over from the 'free' Föhn, being more frequent and easier to recognize. Coding each day with a weather phase (table 2) results in a frequency pattern for observed clinical and physiological deviations. The crucial phase is 3 with 'fine' weather locally and advancing upper clouds and descending air. The observed deviations are frequently as large as they are during the frontal passages of phase 4 and 5, whereas, phase 6, the phase of decreasing shower activity, still locally 'bad', is already free of abnormal human statistics.

Recent routine work at observatories (17, 23), as well as many older studies, show that pressure variations of all frequencies, including infrasound, ozone, air electric potential gradient, and number of ions, sferics, and other natural Hertzian waves are basically undisturbed in 'phase 3' or in 'free' Föhn; air polution is generally below normal. We still lack data on electric space charges.

In conclusion, the complex of weather illnesses which cannot be explained by thermal, hygric, chemical, aerosol, microorganisms or similar environmental factors is more enigmatic than ever. There seem to be more than one critical weather type or, rather, there may be one type critical for each illness. The weather type statistically the most significant shows less clues to the causative agent than any other.

We may, of course, assume that the combined effect of any number of known environmental elements underlies these correlations. Speculations of this type have been offered ever since the difficulty of discovering the one and only cause became obvious. If this 'accord' theory is true, our hopes to solve the riddle from statistical and experimental standpoint and to use our knowledge for cure or prophylaxis are small except for the prediction of Föhn and 'free' Föhn as such.

Some of the above-mentioned observatories make such forecasts of bioclimatic activity routinely. The expected severeness is based on all past experiences. These prognoses are given to physicians at the large university hospitals, sanatoria, etc, not to the public.

Animals seem to respond to the Föhn complex also. The fructose content of bull sperm is 5% above normal during a Föhn (H. Ungeheuer, personal communication). Rabbit's blood clots significantly faster when subtropical air fills the lower and higher troposphere (24); temperature effects can be excluded and fronts show no significant correlation.

d) **Causative Agent Method.** After trivial elements have failed to solve our riddle, all we see from the foregoing is a statistical link between weather complexes and man. The complexes as such cannot hurt man. But something not 'trivial' could go with them. Two such elements were discussed recently, 'sferics' and 'ions'.

Sferics stem from natural electric discharges. These electric wave pulses, especially the longer waves, are transmitted, with the aid of the ionospheric D-layer, for considerable distances.

Emission and transmission depend on the weather, and vary characteristically. The long-wave branches are generally free from man-made emissions, excepting those of long-wave radio emitters. No effects on man living close to 10 km emitters have been reported. Cathode-ray records show sferics as pulses lasting several milliseconds. The pulses are different each instant from wave length to wave length, but the hourly sums of pulses show the same relative pattern for different wave lengths and amplitudes, although the shorter wave group shows certain maxima earlier. Reiter (25) compared his sferics records in the 10 kc area with human data. The statistical N-method was used to compare the deviation from normal of certain events for 2–3 days before, on and after the day having the highest sferics count of the month.

The most interesting events studied are 130,000 auto accidents in Bavaria during a 2½-year period (26). They showed only a poor correlation to frontal passages and Föhn, but were 50% above normal on days having sferics counts exceeding those of the previous and following days, and 50% below normal on days with counts much below neighboring days. These rates are smaller for accidents in Frankfurt, correlated to sferics count in Munich. Similar, but less spectacular increases in pains of amputees, reaction time, birth rate, death rate and industrial accidents are described. A study in Hamburg could not confirm Reiter's results (27). A high frequency of sferics coincides to a certain degree with frontal or 'horizontal-advective' weather (phase 4 and 5), but not with the alpine Föhn and fine weather (23).

The electromagnetic energy applied to man in a sferic is minute. Resonance of any human oscillator to these frequencies is unlikely. Whether sferics are usable only as indicators of weather phases, or whether they themselves act on man remains to be seen. At present no research in this promising field seems to go on.

Geophysical and biological studies have made electric space charge again of interest. Cosmic and terrestrial radiations ionize air molecules, which quickly change into clusters of dozens of air or water molecules. These small ions may then be caught by condensation nuclei, smog, haze, fog or other aerosol particles. The number of ions varies with the origin and the aerosol character of the air and with other factors. A space charge exists if ions of one sign are more abundant.

Dessauer (28) demonstrated substantial effects on man treated with artificial large ions; a negative space charge was beneficial, and a positive generally adverse to health. Bioclimatologists in the twenties argued: could the natural ions do the same and could this explain weather illnesses? There were two objections; free air ion charges were not believed to penetrate buildings, and the Dessauer space charges were 100–1000 times larger than those observed in nature. Both objections may now be discarded.

Parallel indoor and outdoor measurements of small ions in Los Angeles (29, 30) and Switzerland (31) indicate a very close relationship of both. Obviously, ions and, probably, also their space charges diffuse quickly into normal houses. Natural charges of ions may, therefore, be capable of afflicting people indoors.

On the laboratory side, Bisa (32) demonstrated effects on flicker fusion frequency in people exposed to space charges, Worden and Thompson (33) reported increase of proliferation of living cells exposed *in vitro* to negative ions, and many similar observations have been reported.

Using small ions from radioactive sources or from electric discharges and eliminating those of one sign by an electric field, Kornblueh and his co-workers (34, 35) could treat hay fever patients during the pollen season. The space charges ranged from about 1000 to 7000 ions per cm^3. Outside air, containing the usual pollen numbers, was drawn into the chamber. Ample null tests were made. Symptoms were relieved during negative ions exposure, but were not influenced or worsened in positive electric air. The exposures lasted only 30 min. and the relief faded soon thereafter. (For additional data see refs. 36 and 37).

These results focus attention on a rather neglected field of geophysics, the space charges of small ions. Such charges do not necessarily coincide with the total space charge, which comprises small and large ions. Total space charges are more frequently recorded than small-ion space charges. The many data on electrical conductivity are not pertinent since they include the mobility as well as the number of the ions. Generally, positive small ions are more abundant, but fast natural changes of total space charge occurred in Los Angeles (38). The small-ion space charge may vary accordingly.

e) **Test Tube Method.** If sferics, or solar or stellar radio waves, should actually affect man the most likely point of influence would be the

colloidal system. To test this hypothesis, an artificial colloid which has a certain similarity to blood was investigated (39). A 10% Polyvinylpyrrolidon solution in a sealed glass tube was temperature controlled. Its Tyndall-scattering was significantly higher near fronts or with high cyclonic vorticity at the 500 mb level. Similar results have been reported for blood clotting and for the titration value of $Na_2S_2O_3$ in 2% KI (40). Cosmic influences on anorganic, sealed colloids have been claimed and are supposed to occur simultaneously in Italy, Austria and Belgium (41–43). Whether we have to add a biocosmology to our field remains to be seen.

CONCLUSION AND SUMMARY

The long search for the reality and cause of weather illnesses has led to the establishment of two weather types which are potentially harmful. One is the long-suspected frontal or disturbed weather, and the other, the 'free' Föhn with descending subtropical air in the whole troposphere. Each type correlates with a specific set of clinical and physiological events. Trivial surface weather elements such as pressure, temperature, wind or humidity are not the likely causes. Sferics seem to be indicators of weather rather than causes of illness or accident. Successful treatment of hay fever with artificially negatively charged air might give a clue to the explanation of weather effects.

After conclusion of this manuscript an interesting book about meteorotropism and its pharmacological influences was received (44).

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AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Symposium on Studies of Antibody Formation Following Cellular Transfer[1]

Chairman: MERRILL W. CHASE

INTRODUCTORY REMARKS

MERRILL W. CHASE

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THAT IMMUNOLOGISTS ordinarily deal only with the ultimate product of the antibody-forming apparatus is understood generally and accepted. Nonetheless, circumstances force us away from the older position of treating antibodies as a single group. The varieties newly recognized among antibodies—non-precipitating and non-skin sensitizing, non-precipitating and skin sensitizing, Rh antibodies capable of combining in the diluted state only when the dilution is made in media containing proteins, antibodies of low avidity, antibodies from a single species endowed with different half-lives, to mention but a few—only serve to rekindle our interest in observations that are, preliminarily, qualitative in nature. During this same period, attention has been turned backward to the genesis of antibodies, by observation on changes occurring in mononuclear cells of the spleen and lymph nodes during initial antigenic stimulation, by investigation of splenic and non-splenic sources of antibody production *in vivo*, by studies on antibody production in tissue culture, by various experiments in which radio-tagged amino acids have been incorporated into antibody during its *in vitro* synthesis. In such an era of serological renaissance, reviews dealing with various segments of current work can prove useful; the explorations to date seem worth recounting. One fact is regrettable, namely, that the time available for the Society's symposium at the Federation Meeting limits the number of panelists so that several who are making principal contributions in this field are not represented. We are only too much conscious of the omission of direct reports by Drs. Taliaferro, Stoner, Thorbecke, who is visiting in this country, Stevens, and several other chief contributors at home and abroad. The fields of inquiry explored by Dr. Ehrich and by Drs. Dougherty and White would also be useful to recount here.

Antibody formation following transfer of a suspension of single cells from one individual to another, antigen being supplied at some step in the chain of operations, harks back to a fundamental observation made by Topley, who noted the swift appearance of agglutinins in a rabbit receiving bits of chopped spleen from another rabbit that had been lightly stimulated by an injection of a bacterial vaccine. The recipient rabbit did not experience a direct stimulus by antigen, for it was unable to give later a response of the 'secondary' type. Topley's hypothesis was that he had transferred an 'intermediate' antigen capable of inducing speedy antibody formation but one that could not stimulate the animal to recognize the original and full bacterial antigen. The observation was allowed to lapse, and this particular hypothesis was not recounted in certain editions of Topley and Wilson's textbook.

Quite another type of laboratory effort focused attention on Topley's phenomenon. This was the finding that suspensions composed of single cells could transfer the delayed-type of skin hypersensitivity from sensitized guinea pigs to normal guinea pigs. Sensitization induced by various simple chemical allergens, and tuberculin-type sensitization (induced by killed *Myco. tuberculosis* injected in paraffin oil) could both be so transferred. Still later, despite the complete absence of information relating dermal hypersensitivity of

[1] Atlantic City, N. J., April 18, 1956.

delayed type to any role played by circulating antibody, it was found that the injection of suspensions of single cells from antibody-producing guinea pigs could result in the appearance of antibody in the circulation of recipient normal guinea pigs. Rapidly, the latter approach was transferred to studies using the rabbit, and the procedure was found to possess general validity. It is with the latter observation that we are concerned tonight, not with the cellular transfer of dermal hypersensitivity.

It will not be out of place, however, to recount here the considerable degree of parallelism in cell sources, in specificity, in the limitation of transfer within the species that ordinarily characterize both varieties of cellular transfer. More, the finding that the delayed-type of hypersensitivity can be transferred in man by means of lysed cells places the cells of the new host in a functional role. No such event has yet been described for the cellular transfer that leads to the appearance of circulating antibody. The emphasis has, rather, been confined to this question: whether the appearance of antibody reflects the metabolism of the introduced cells in a culture medium provided by their new host. An affirmative answer to this question is not viewed by your chairman as having unquestionably validity. Experiments in which organized populations of cells, e.g. minced spleens, lymph nodes and the like, are found to synthesize antibody *in vitro* are not surely to be trusted as an adequate model, in view of the cellular complexity of the bits of whole tissue.

These opening statements may be concluded by drawing attention to the several pieces of evidence in which an early appearance of antibody is detected following antigenic stimulation. Oerskov's work with cowpox vaccine pointed to the appearance of antibody in 2 or 3 days, and a similar time has been shown adequate after the injection of pneumococci. In Campbell's so-called diaphelic immunization, in which bacterial vaccines are instilled into the udder of lactating, normal cows, a very early appearance of antibody in the milk has been reported. As concerns the events occurring after secondary stimulation, the reports of Wesslén with lymph cells in roller-tube culture and of Stevens with splenic fragments (as heard in the paper reported today) suggest that antibody may be synthesized with unexpected promptitude.

It seems certain that immunology, aided by immunochemistry and biochemistry, is breaking out of its former confines, and that it will attract an increasing number of investigators who will have at heart the theoretic rather than the utilitarian aspects of the subject.

QUANTITY AND QUALITY OF DIPHTHERIA ANTITOXIN APPEARING AFTER TRANSFER OF CELLS TAKEN FROM IMMUNIZED RABBITS

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THE FINDING that circulating antibody would appear in recipient, normal guinea pigs owing to the injection of white cells taken from sensitized guinea pigs was reported before the American Association of Immunologists at its annual meeting in 1950 and was published later (1, 2). In this instance, the donor animals possessed both a delayed allergy (with respect to contact with picryl chloride) and a content of circulating antibodies reacting specifically with picrylated proteins *in vitro*. Following the injection of 0.6–0.7 ml of washed cells from the spleens of exsanguinated donors, 0.45 ml being introduced by the intravenous route and the remainder by the intraperitoneal route, the appearance of antibody in the recipient animals was detectable both *in vitro* (Schultz-Dale reactions *vs.* picrylated protein) and *in vivo* (injection of serum into the skin of normal guinea pigs (cf. 3) and introduction of picrylated protein intravenously 17 hr. later, the basic part of the procedure that is now known as Passive Cutaneous Anaphylaxis). The transfer of cells teased from lymph nodes of the donors led likewise to the appearance of antibody in the circulation. The antibody was detectable between

48 and 72 hr. after transfer of cells (sometimes at 24 hr.), and increased in amount for the following 4 or 5 days, and then disappeared in a course consistent with that representing a typical half-life slope. It was established that damage to the cells of either splenic or nodal sources, as by freezing and thawing, or heating to 48°C for 15 min., would entirely prevent the appearance of antibody. In conformity with this, extracts of cells were shown not to contain demonstrable amounts of pre-formed antibody, nor could they stimulate the formation of new antibody. Moreover, the stimulus that the cells provided to the recipient animals for inducing the appearance of antibody did not appear to represent a primary antigenic stimulus. The cells appeared to be mature lymphocytes.

At this point, some attempts were made to use the cells of animals that did not possess a delayed-type allergy, but were solely producers of circulating antibody. In the hands of one of us, hemolysins for sheep cells were found to appear (in low concentration) in normal rabbits that had received cells from specifically prepared donor rabbits (1). Dr. and Mrs. T. N. Harris explored this field with a superior system and made more convincing observations (4, 5).

Because normal rabbits can possess low-grade hemolysins for sheep cells and agglutinins for Gram-negative bacteria, we felt some uncertainty with regard to the type of stimulus that the cells were providing in instances in which increases in titer had to be observed. It seemed to us necessary to investigate cases in which antibody had to arise *de novo*, and to quantitate the amount of antibody accurately. With Dr. Wager, we undertook a study of the diphtheric toxin-antitoxin system, for antitoxin is not found in normal rabbits and quantitation may be made accurately, the amount of antibody being measurable to ±5% if one so desires. For this, bleedings were made at selected times and the ability of the sera to neutralize the Cohn-Pappenheimer purified diphtheria toxin was examined by injecting graded mixtures of the two reagents into the skin of normal rabbits. A given specimen of antitoxic serum can be equated with officially standardized diphtheria antitoxin so that its ability to neutralize the necrotizing effect of diphtheric toxin in skin can be expressed in terms of known antitoxin units (AU) per ml. Conversion of AU/ml to milligrams of antibody-nitrogen per ml is based on the relation: 1 AU = 2.5 μg of antibody-nitrogen.

The validity of the operation here outlined is subject to one limitation, as Jerne (6) has so ably and thoroughly pointed out, namely, the diphtheric antitoxin in the unknown specimen must possess a high degree of avidity in combining with diphtheric toxin, similar to that which characterizes the officially distributed standard diphtheria antitoxins. The first outpouring of antitoxin by an animal will not possess a high degree of avidity, hence a simple comparison of such a serum with standard antitoxin will not be sufficient. Nonetheless, the degree of avidity of an antitoxic serum can be measured, its actual amount of neutralizing antibody can be determined and, where necessary, a corrected expression of AU/ml can be obtained. In the following, attention has been paid to the question of avidity, and proper values for AU/ml are offered in the tables. (The detailed procedure developed in this laboratory, an extension of the prinicples developed by Jerne, will be presented elsewhere. It will be adequate to point out that the measurement consists of the difference between the neutralizing capacity of an antitoxin as determined under *a*) nearly optimal conditions and *b*) suboptimal conditions. Sera of high avidity, such as N.I.H. Standard Antitoxin, exhibit a four-fold difference in the two tests; sera of low avidity, on the other hand, may exhibit differences as great as 32- to 64-fold. Such differences can be shown to represent fixed properties of diphtheric antitoxins.)

Two fundamental types of experiment were undertaken by Wager and Chase (7). In the first, rather large amounts of toxoid adsorbed to alumina cream (1–3 injections of 435 Lf or a total of 0.2–0.6 mg antigen-nitrogen) were injected intracutaneously and intramuscularly and the animals were sacrificed within a week. Splenic cells and lymph node cells were secured by teasing under fluid, and these were injected intravenously into normal rabbits. Diphtheria antitoxin appeared in most of these recipients within 3 days. In some of the recipients, two separate outpourings of antibody were noted, the initial one seen regularly and completed in from 3 to 9 days, and a later rise in antitoxin commencing about the 16th day and continuing with all the characteristics of an active sensitization. Evidently, cells could be harvested on occasion that possessed sufficient toxoid to stimulate the antibody-forming apparatus directly.[1] Indeed, the two bursts of antibody

[1] The presence of contaminating antigen intro-

could be separated: *1*) when only the early burst of antibody appeared, reinjection of toxoid 120 days later would elicit no secondary response; *2*) when the cells were killed by being held for ½ hour in Hanks' solution that had been saturated with chloroform and freed of excess chloroform, the killed cells failed to produce the early burst of antibody, but incited the late burst and primed the recipients to undergo a specific secondary response by means of a 'booster' injection.

In the second type of experiment, rabbits were primed with 50 Lf of fluid toxoid (23 μg of toxoid-nitrogen) on a single occasion, then were restimulated on day 90 with an injection of 200 Lf toxoid adsorbed on alumina cream, given intravenously. The animals were sacrificed on the 3rd or 4th following day, and transfers of splenic and lymph node cells were made to new rabbits. Here, only the first burst of antitoxin was seen, and the rabbits were not primed by the injected cells to undergo a secondary response when toxoid was injected 6 months later. Results of the two types of experiment are given in the lower part of table 1.

It was apparent, however, that the property of avidity could be used to explore the question whether antitoxin that arose in the recipient within the first few days stemmed from the stimulus of some special antigen present within the cells or was a reflection of the avidity-type of antibody that was being produced currently by the cell donors. If the latter were the case, production of antibody *de novo* would be shown, either effected by the transferred cells themselves or through the recipient's utilization of some antibody-directing material contained within the cells. Examination of the avidity of antibody in various donors-and-recipient rabbit groups was most encouraging, hence critical experiments were undertaken in guinea pigs. As has been reported (cf. 6), the change in avidity with increasing number of antigenic stimuli is more gradual in the guinea pig than is seen in rabbits. Groups of guinea pigs were therefore prepared with different routes of injection and numbers of preparatory injections, and then were rested variously for 25–330 days. Upon restimulation, the antitoxin that each group produced increased in avidity to some degree, as expected, yet the conditions had been chosen so that clear differences in avidity were present in the antitoxins produced by the animals of each group. In this way, at least three and probably four demonstrably different qualities of antitoxin were produced, differing respectively by 5-fold, 8-fold, 16- to 18-fold and 32-fold increases in the amounts of antibody required to neutralize toxin under sub-optimal conditions as compared with optimal conditions. Cell transfers were made from each group of guinea pigs to new recipients, and careful studies were made of the avidities of the antitoxins present in cell donors and cell recipients. It was found (8, 9) that the antitoxin appearing in a recipient guinea pig was constant with respect to avidity throughout the course of its accumulation, and that the particular avidity or quality of antitoxin that was produced in a recipient animal always duplicated the quality of the donor's antitoxin. Some evidence for even one further detail was obtained: as mentioned above, each new antigenic stimulus was destined to drive the avidity level slightly upwards; correspondingly, the avidity of the recipient's antitoxin was found to be the same as that of the donor group not at the time of their sacrifice (3 days after the restimulation) but rather at the time of their maximum new accumulation of antibody (6 days after restimulation). One may therefore conclude that the antibody found after cellular transfer was not incited actively by means of a special form of antigen contained within the cells (the varieties of antigen needed to account for the various degree of avidity would require special hypotheses), but resulted from the donation of some element (template for antibody formation? enzyme? vital synthetic capacity of the donated cells themselves?) that is remote from the actual antigen. This element evidently bears the stamp of the specific previous antigenic stimulation and will determine the formation of antibody. That active cells cannot induce the appearance of antibody in another species (guinea pig cells into rabbits, rabbit cells into guinea pigs, table 2) also speaks against an hypothesis of an intermediate antigen contained within the cells. Evidently in the transfer of cells from restimulated donors we have to do with a later step in the course of antibody production than Dr. Harris is dealing with when he mixes Shigella extracts and normal rabbit lymph

duced *via* the cells has been met by Harris and Harris also (5). In their case, active antibody-formation in response to *Shigella* antigens has been so rapid that it has been necessary to use as recipients rabbits that have been rendered relatively inert to stimulation by antigen by means of x-ray treatment. We ourselves were fortunate in that the onset of demonstrable antibody-formation to diphtheric toxoid occurs later and allows observation of both events independently.

TABLE 1. ANTITOXIN FOLLOWING TRANSFER OF LYMPH NODE (L) AND SPLENIC CELLS (S)—ANTIBODY-NITROGEN (mcg/ml) IN RECIPIENTS' SERUM (CORRECTED FOR AVIDITY)

Exp.	Cells i.p.	Ab-N/ml. (cells)	Days following transfer 2	4	5	7	8	11	15	21	35	Recall (127-140)
G.P. → G.P.												
13-B*	L 0.42 ml.(10)	.005	.2	1.0‖								
	S 0.5 ml.(11)			1.6		2.4			1.6	1.0	.2	†
13-A*	L 0.5 ml.(8)	.004	.24	1.6		5.1		5.1	4.5	3.2	.8	†
	S 0.7 ml.(10)			.3		.6		.6	.4	.3		No
14-A*	L 0.76 ml.(8)		.65	3.6		5.1		4.1	2.0	.8‖		
	L 0.76 ml.(8)		.4	2.8		5.1		4.1	2.0	1.2		No
	S 0.82 ml.(9)		.03	.1		.2		.2	.2			No
12-B	L 0.5 ml.(8.6)	⩽.015			4.7		4.7 ‖					
	S 0.95 ml.(11)				1.0		1.0 ‖					
7	L 0.63 ml.(8.6)	.037			31.2		36.4 ‖					
	S 1.1 ml.(11)				6.5		7.8 ‖					
11-A	L 0.45 ml.(7.5)		.02+	.4		2.6		4.1		3.2	1.8	No(?)
	S 0.52 ml.(11)			.08		.8		3.2		3.2	.8	No
	L 0.45 ml.(7.5) ‡		0	0		0		0		0		No
Rab. → Rab.				3 d.								
4-B*	L 1.8 ml.(2.5)		.1	.2+	1.0	.8		bimodal curve				Yes
4-A*	L 1.5 ml.(2.5)		.4	.8	1.6	.7		bimodal curve				Yes
OAW 6	L 0.7 ml.(2.6)	.004		.3	.8	.5		.35		.15		No

*Correction for avidity by factor 1.57. ‡Guinea Pig → Rabbit.

TABLE 2. TOTAL ANTITOXIN RECOVERED* AFTER TRANSFER OF LYMPH NODE CELLS (Ab-N, MICROGRAMS)

Exp.		Cell Volume		Within Cells	Recipient	Days after Transfer
G.P. → G.P.	XIII-B†	0.42 ml.	(10 D)	.06	12.7	4, killed.
	XIII-A†	0.5 ml.	(8 D)	.045	58.5	7 - 11
	XIV-A†	0.76 ml.	(8 D)		57.5	7
	XIV-A†	0.76 ml.	(8 D)		56.0	7
	XII -B	0.5 ml.	(8.6 D)	.17	51.6	5 - 8. killed.
	VII	0.63 ml.	(8.6D)	.52	510.0	8, killed.
	XI -A	0.45 ml.	(7.5D)		41.0	11
G.P. → Rab.	XI -A	0.45 ml.	(7.5D)		0	2 - 120
Rab. → Rab.	II†	0.8 ml.		nil		
	IV -B†	1.8 ml.	(2.5D)		82.5	5
	IV -A†	1.5 ml.	(2.5D)		127.0	5
	OAW 6	0.7 ml.	(2.6D)	.36	68.0	5
Rab. → G.P.	$IV\text{-}B_1$	0.45 ml.	(0.65D)		0	3 - 60
	$IV\text{-}B_2$	0.44 ml.	(0.8 D)		0	3 - 60

*Not corrected for distribution between serum and tissues; factor = ca. 2.5 for both species.

†Corrected for non-avidity of antitoxin.

node cells *in vitro* prior to injection into a recipient rabbit.

The amount of antitoxin that has been found following the transfer of lymph node and splenic cells is contained in table 1, being here expressed as micrograms of antibody-nitrogen per ml of serum, and in table 2, expressed as total micrograms of antibody-nitrogen recovered in the serum after the transfer of lymph node cells. Owing to the distribution of antibody between cells and tissues, the figures presented must be multiplied by a factor of *circa* 2.5 (obtained experimentally by infusion of rabbit antitoxin into normal rabbits) to obtain total amounts of antibody produced. It will be seen that, at times, tiny amounts of pre-formed antitoxin may be found within the cells transferred; one wonders in what measure such pre-formed antibody may have been responsible for certain interpretations that have been arrived at by examining restimulated lymph nodes by Coons' fluorescein-labeling technique? The amount is indeed small, and bears little relation to the amount of antitoxin produced by means of the transfer of these cells and found free in the blood stream (table 2), which is 200-1300 times greater than that which may sometimes be detected within the cells; at times, no antitoxin at all may be present within the injected cells. Further, as stated above, the true amount of antibody produced is about 2.5 times greater than that which remains in the serum at the time of optimal concentration. No reliable

proportionality exists between either the volume of cells transferred, or the contribution per donor, with the resultant amount of antibody-nitrogen obtained in the serum. With regard to transfers of lymph node cells within the guinea pig species, approximately 100 μg of antibody-nitrogen per 1 ml cells transferred was observed in the serum on three occasions, but values respectively one-third and eight times as large have been secured. The latter value (810 μg/ml cells) indeed exceeds the amount of variation that could be expected from the fact that cells from stimulated lymph nodes are diluted to various degrees by inert cells from other nodal sources. It is suggestive, rather, of a superior utilization of the transferred cells by the recipient animal, perhaps on the basis of a greater degree of genetic compatibility. In terms of micrograms of antibody-nitrogen contributed to the serum by lymph node cells per cell-donating guinea pig, 5.5–7.3 μg is the mid-range of our experiences, with values respectively one-fifth and 8 times as large being encountered. The order of magnitude is shifted in the case of transfers of rabbit cells within rabbits. Values approximately 100 μg of antibody-nitrogen per 1 ml cells have been obtained in the serum on two occasions, but the proportionate contribution per donor to the antibody in the serum on these two occasions were 51 and 26 μg respectively, values which cast further doubt on proportionality calculations.

From the above studies, a fairly clear idea may be got of the amount of antibody that can be secured by the transfer of splenic and nodal cells, taken on the 3rd and 4th day after restimulation of donors, but not subjected to further contact with antigen in the recipient animal as Roberts and Dixon (10) have practiced. The antibody was measured as actual neutralizing antitoxin, and allowance was made for its state of avidity. Values of the general order have been secured by Stavitsky on diphtheric antitoxins by means of the Boyden tanned-erythrocyte hemagglutination technique, although impurities in the toxoid preparations used to coat the tanned cells and the presence of antibodies against non-toxoid impurities in the antitoxic sera appear to make the results rather less reliable. The work further emphasizes the parameter of avidity, which must play a more significant role than has been commonly assumed in describing a first output of antibody. That the common use of the rabbit by immunologists for the production of antibody may have blunted the need for recognizing differences in avidity (since, as quoted by Jerne, [6], the rabbit comes to produce antibody of high avidity under the impetus of a relatively short series of stimuli by antigen) does not lessen the problem that confronts those who study the genesis of antibody.

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FORMATION OF AGGLUTININS TO SHIGELLA PARADYSENTERIAE BY TRANSFER OF LYMPH NODE CELLS[1]

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In recent years the transfer of cells and tissues from one animal to another has become one of the fruitful approaches to the study of antibody formation. This development stems most directly from a series of studies by Landsteiner and Chase (1) and then by Chase (2) in the passive transfer by cell of delayed hypersensitivity. In 1950 Chase (3) reported that the transfer of cells of lymph

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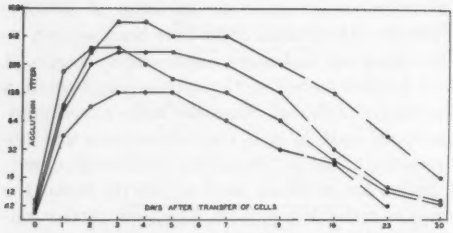

FIG. 1. Appearance of agglutinins to dysentery bacilli in the sera of rabbits following the intravenous injection of 225 million cells obtained from lymph nodes of donor rabbits 4 days after the injection of dysentery bacilli. (Reproduced from the *Ann. New York Acad. Sc.*, with permission from the Academy.)

nodes and spleens of guinea pigs highly sensitive to picryl chloride to normal guinea pigs led to the development of skin sensitivity and anaphylactogenic antibodies to this agent in the recipient animals.

The technic of cell transfer described by Chase was adapted in our laboratory to the study of antibody formation in a system characterized as follows: the cells were those obtained from lymph nodes of the rabbit; the immunologic response was a primary one; the material used as source of antigenic material was the whole dysentery organism, *Shigella paradysenteriae*, or soluble antigenic material derived thereform; and the test for antibody in the serum of the recipient animal was agglutination of the whole *Shigella*. In this system the primary response is rapid and, relative to the sensitivity of the agglutination test, of adequate magnitude for the study of this response.

1) Transfer of Lymph Node Cells 4 Days After the Regional Injection of Antigen. From previous study of rabbit popliteal lymph nodes draining sites of injection of dysentery bacilli, data were available as to the intervals after injection of antigen that antibody appeared and reached its maximum concentration in this node. Accordingly, dysentery bacilli were injected into the hind feet of rabbits, and at a time when it was known that the concentration of agglutinin within the node was reaching its peak, *i.e.* 4 days later, the nodes were excised. Cells obtained by teasing the lymph nodes were washed and transferred to fresh recipients. Serum was obtained from the recipients at various intervals after transfer and tested for the presence of agglutinins to dysentery bacilli. It was found, as can be seen in figure 1, that agglutinins appeared on the first day after transfer, generally reached a maximum concentration by the third day, and began to decline shortly thereafter (4).

2) Effect of Injury to the Cells Transferred. A question of prime importance was whether the appearance of agglutinins in the sera of recipients after the transfer of the lymph node cells could be attributed either to the transfer of pre-formed antibody, or to antibody formation by the tissues of the recipient animal as a result of antigen transferred in the cell suspension. In this connection experiments were performed in which portions of the lymph node cell suspensions were subjected to various injurious treatments prior to transfer and the agglutinin titers were compared in recipients of injured and control (fresh) cells. In such experiments the cells were frozen and thawed 3 times alternately in a dry ice and alcohol bath, or irradiated in the ultraviolet range, or exposed to iodoacetate at a concentration of 10^{-2} or 10^{-3} M, or incubated at 37°C for 24 hr. As can be seen in figure 2, after the transfer of the control suspensions (fresh cells) antibody appeared in a pattern similar to that shown in figure 1. Following the transfer of injured cells, however, agglutinins did not appear at our threshold of measurement at all, or appeared at a later time relative to the time of their appearance after the transfer of fresh cells, and in low titer (4). The later appearance of agglutinins has been attributed to antibody formation by the tissues of the recipient in response to antigenic material in the cell suspension. The fact that as mild a treatment of the lymph node cells as incubation at 37°C for 24 hr. resulted in a failure of agglutinins to appear in the first few days after transfer, in contrast to the results of transfer of fresh cells, indicated that viable cells were necessary for this effect. Additional evidence that the antibody found in the recipients' sera was not due to release of preformed antibody, or to effects of transferred antigen was provided by the dissimilarity between the curves relating antibody concentration to time in cell transfer experiments and those found either in active or passive 'immunization', the finding that the amount of agglutinin extractable from the cell suspension transferred was only a few per cent of that measured subsequently in the serum of the recipient, and the observation that the various forms of treatment to which the cell suspensions were subjected did not measurably

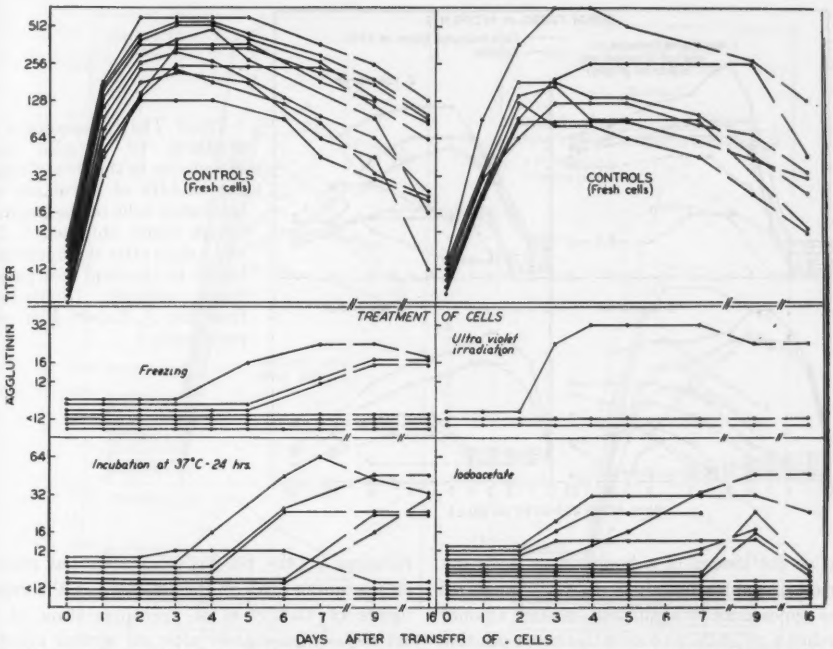

FIG. 2. Agglutinin titers in recipient rabbits following transfer of lymph node cells subjected to various injurious treatments in comparison with untreated control cells.

affect either the antigenicity of the *Shigella* agglutinogen or the reactivity of the agglutinin.

3) Variations in Interval Between Injection of Antigen into the Donor and Collection of its Cells. Because of the indications that a function of the transferred cells was probably involved in the production of antibody in the recipient, it was of interest to determine how soon after the injection of dysentery bacilli into the foot pad the lymph node cells would be effective in transfer. Data from such experiments are shown in figure 3. When cells obtained 3 days after the injection of antigen into the donor were transferred to recipients, the resulting antibody-time curves resembled those obtained with 4-day cells. After the transfer of fresh cells obtained at a 2-day interval, antibody appeared on the second day after transfer, and in the case of cells obtained at a 1-day interval, agglutinins appeared on the 3rd day after transfer. In each case the transfer of cells incubated at 37°C for 24 hr. served as a control. In experiments involving a 2-day donor interval (the interval between injection of antigen into the donor and the collection of its cells) it was still possible to differentiate between the effect of transfer and that of active 'immunization' by antigen present in the cell suspension. In the case of the 1-day donor interval this differentiation was not possible, since agglutinins appeared on the third day after the transfer either of fresh cells or of heated cells. In order to determine whether activity of the transferred cells or active 'immunization' of the recipient could account for the agglutinins found in recipients of 1-day cells, such recipients were subjected to deep roentgen irradiation 24 hr. before cell transfer. This procedure served to suppress the ability of the tissues of the recipient to form antibodies and did not reduce the concentration of agglutinins found after the transfer of fresh cells. Following the transfer of fresh 1-day cells to irradiated recipients, agglutinins appeared on the third day, whereas after the transfer of incubated or heated 1-day cells agglutinins did not generally appear within the first week (5).

These observations suggested that following the introduction of dysentery bacilli into the hind foot pads of rabbits the production of agglutinins to these organisms in the regional lymph node was a continuous process, which, if interrupted in the tissues of the host, could be con-

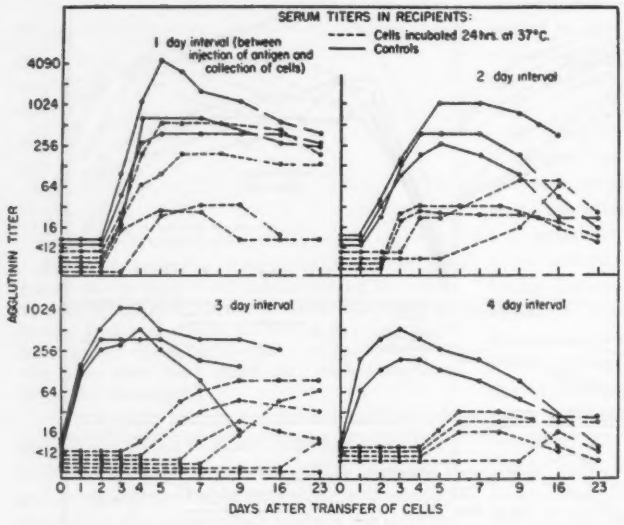

FIG. 3. The appearance of agglutinins to *Shigella paradysenteriae* in the sera of recipient rabbits of untreated and incubated cells of the popliteal lymph nodes obtained 1, 2, 3 and 4 days after the injection of bacilli in the hind foot pads of donor rabbits. (Reproduced from the *J. Exper. Med.* with permission.)

tinued in the tissues of a homologous recipient. A period of approximately 3 days was required for the appearance of agglutinin, in such amount as to reach a measurable concentration on dilution in the serum of the 1-kg recipient, and such fraction of that time as had not been spent in the donor rabbit was required in the recipient rabbit. This was again indicated when the donor interval was further reduced. In experiments involving donor intervals between 16 hr. and 10 min. agglutinins were detected in the sera of irradiated recipients, usually on the fourth day after transfer.

This finding resembles the observation of Oakley *et al.* (6), who injected alum-precipitated diphtheria toxoid into the interscapular fat of rabbits and then transplated this tissue to other rabbits. It was found that the time of appearance and maximum titer of diphtheria antitoxin in the recipient host varied with the interval between the second injection of the antigen and the transplantation. (In this system antitoxin appears, in the secondary response of ordinary active 'immunization', 3 days after the secondary injection of toxoid, and reaches a maximum on the 10th.) In the transplantation experiments, if the fat tissue was obtained 10 days after the second injection of antigen, antitoxin appeared and was at its maximum concentration in the serum of the recipient on the 1st day after transfer. If the fat tissue was obtained only 3 days after the injection of antigen into the donor, antitoxin appeared in the serum of the recipient on the 1st day thereafter but reached its maximum level on the 7th day. In the experiments of Oakley *et al.* transplantation of the fat tissue immediately after its second injection with antigen did not lead to the appearance of antitoxin in the pattern described. The authors concluded that this was so because no time had been allowed for infiltration of the injected mass of fat, while *in situ*, with cells which could produce antitoxin.

4) *In Vitro* Contact of Lymph Node Cells with Antigens of *Shigella paradysenteriae* in Various Forms. In the experiments involving variations of the donor interval, as described above, it had been found that cells obtained from a donor animal sacrificed 10 min. after injection of the *Shigella* could be transferred to irradiated recipient animals with the subsequent appearance of agglutinins in the sera of the latter. In later experiments cells were obtained from uninjected donor rabbits and incubated *in vitro* with suspensions of *Shigella*. Such suspensions were washed and transferred to irradiated rabbits. The sera of the latter were found to contain a measurable concentration of agglutinin on the 4th day. This concentration rose, to reach a maximum on the 6th–8th day after cell transfer, and then began to decline. Irradiated recipients of heated portions of such cell suspensions rarely showed measurable agglutinin titers within the 1st week (7).

In the next study use was made of a soluble form of antigenic material derived from the

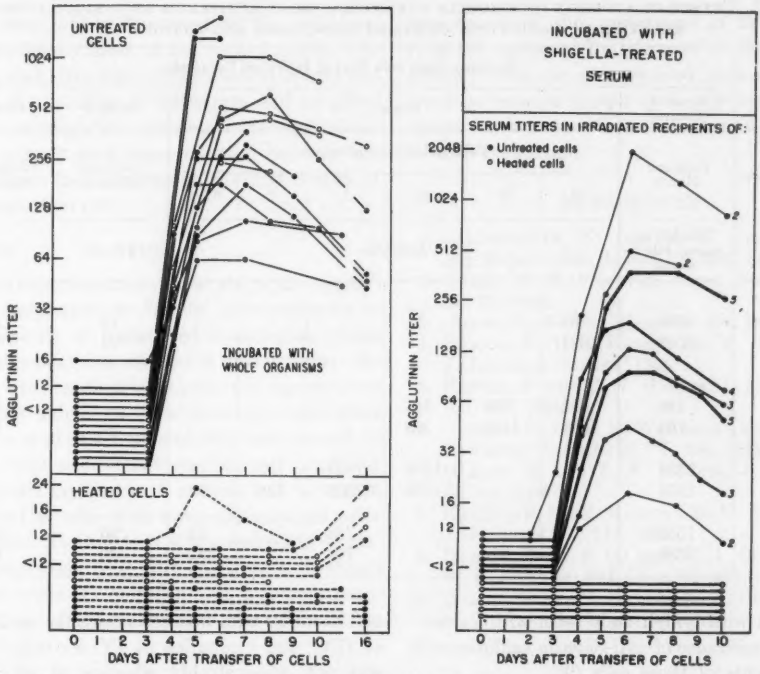

FIG. 4. Appearance of agglutinins in irradiated recipients of lymph node cells which had been obtained from normal donor rabbits and then incubated *in vitro* with suspensions of whole *Shigella* (left half of figure), or with *Shigella*-treated rabbit serum (right half). The lower part of each half shows agglutinin titers of irradiated recipients of heated portions of such cell suspensions. (Reproduced from the *Ann. New York Acad. Sc.* with permission from the Academy.)

Shigella. This was obtained by incubating the organisms in normal rabbit serum at 37°C and then removing the bacterial cells by centrifugation and Seitz filtration. The resulting filtrates gave serologic evidence for the presence of antigenic material. If cells from normal rabbits were incubated *in vitro* wich such *Shigella*-treated serum and then washed and transferred to irradiated recipients, agglutinins appeared in the sera of the latter as in the case of the *in vitro* incubation with the whole organisms, with the exception that agglutinins occasionally appeared at measurable concentration by the 3rd day (8). Typical curves of serum agglutinin titer versus time are shown in figure 4, for recipients of cells incubated *in vitro* with whole *Shigella* or with *Shigella*-treated serum.

In the case of *in vitro* incubation with the soluble form of the antigen, there was evidence of very little excess antigen in the suspension, since the transfer of heated suspensions of incubated cells to non-irradiated animals did not result in the appearance of agglutinins in half of the animals so tested. It was thus possible to demonstrate the cell-transfer effect by the use of normal rabbits as well as irradiated ones.

More recently a soluble form of antigenic material has been prepared for cell-transfer studies by trypsin treatment, a procedure which yields material with several advantages over the *Shigella*-treated serum. Following the treatment with trypsin, the bacteria-free filtrates gave serologic evidence for the presence of antigenic material that reacts with anti-whole *Shigella* serum. These filtrates were incubated *in vitro* with lymph node cells from normal rabbits, and the sera of irradiated recipients of such cells were found to contain agglutinins to *Shigella*, in the same pattern as that found in the experiments with the *Shigella*-treated serum. The filtrates of trypsin-treated suspensions of *Shigella* were dialyzed in the course of preparation for use in the cell-transfer experiments. It was also found that the dialysates of such filtrates could be used for

TABLE 1. EFFECT OF ADDITION OF SHIGELLA PARADYSENTERIAE ANTISERUM AT VARIOUS TIMES IN RELATION TO INCUBATION OF LYMPH NODE CELLS AND ANTIGEN

	Maximum Agglutinin Titer of Individual Recipients								
	Group 1 (Control)	Group 2						Group 3	Group 4
First incubation	Cells and filtrate	Cells and filtrate (minutes)						Cells and Antiserum	Filtrate and antiserum
		30	20	10	5	2	0.5–1		
Second incubation	Tyrode's or normal rabbit serum	Antiserum						Filtrate	Cells
Exper. no.									
421	1536	768						12	16
	2048	1024						24	64
									16
433	768	384	768	384					12
	768	1024	1536	768					16
439	1536			1536	1024	192	48		
	1536			1536	1536	768	96		
443	1536				768		32	96	64
	1536				768		192	32	12

incubation with lymph node cells, with the subsequent appearance of anti-*Shigella* agglutinins in the recipients of those cells (9).

5) Evidence for Occurrence *in Vitro* of an Initial Reaction Between Lymph Node Cells and Antigen. The introduction of soluble forms of the antigenic material derived from *Shigella paradysenteriae* in these studies has made possible better evaluation of the role of the *in vitro* incubation in initiating the subsequent formation of agglutinin in irradiated recipients. First, as might be expected, the use of soluble forms of the antigen permits more effective washing away of excess antigen from the lymph node cells after the incubation. A sensitive test for such excess antigen in the cell suspension is provided by the injection of heated portions of the cell suspensions into non-irradiated recipients. In the experiments involving filtrates of trypsin-treated bacilli there is evidence that very little antigen is carried over with the cells, since at a concentration of 10^{-4} almost all normal recipients of heated cells fail to develop measurable levels of agglutinins, although the use of this concentration leads to the development of maximal agglutinin titers in irradiated recipients of fresh cells. Second, in the study with *Shigella*-treated serum it was found that variations in the conditions of the *in vitro* incubation of cells and antigen were reflected in the serum agglutinin titers of recipients. Thus, incubation of cells and filtrate at 4°C for 30 min. was not as effective as 20 min. at 37°C, and incubation at 4°C for only 10 min. was not demonstrably effective at all. Third, recent data on the effect of anti-*Shigella* serum on the incubation of lymph node cells with filtrates of trypsin-treated dysentery bacilli have supported the hypothesis that an initial reaction occurs *in vitro* between cell and antigen. Some of these experiments are summarized in table 1. In this table it can be seen that whereas the usual incubation of cells with antigen before transfer yielded agglutinin titers such as those shown in the first column (*group 1*), incubation of the filtrate with antiserum in excess before the addition of the cells caused a marked decrease in the recipients' agglutinin titer after the cell transfer (*group 4*). Similar results were obtained when the antiserum was added at the same concentration to the cells before the addition of the filtrate to the incubation mixture (*group 3*). If, however, cells and filtrate were incubated for the usual period of 30 min. at 37°C, the subsequent addition of antiserum did not cause a reduction in the agglutinin titers of the recipients (*group 2*). In subsequent experiments lymph node cells were incubated with filtrates, and after various times of incubation (30–0.5 min.) antiserum was added and the total mixture further incubated. It was found that even when the antiserum was added as soon as 5 min. after the beginning of the incubation of cells and filtrate the level of serum agglu-

tinin titers which developed in the recipients were similar to those of the control group. The finding that after 5 min. of incubation of cells and filtrate the addition of antiserum had no effect on the subsequent appearance of agglutinins would suggest that some reaction between cell and antigen had occurred in the first 5 min. of that incubation (10).

SUMMARY

In the experiments reviewed above, the appearance of agglutinin to *Shigella paradysenteriae* in the recipients of transferred homologous lymph node cells has been studied in two systems: the first, in which antigenic material was injected into the donor animal and the draining lymph node was then used as a source of cells and, second, in which lymph node cells were obtained from donor animals not injected with antigen and were then incubated *in vitro* with antigenic material prior to transfer. The relation of the recipients' agglutinin titers to the time after cell transfer is shown for both types of experiments. It is demonstrated that injury of the transferred cells prevents the appearance of the agglutinins in the sera of recipient animals. In the case of the *in vitro* system, evidence is presented that an initial reaction between lymph node cells and antigen occurs within the first few minutes of the *in vitro* incubation.

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QUANTITATIVE ASPECTS OF ANTIBODY RESPONSES OF TRANSFERRED LYMPH NODE AND PERITONEAL EXUDATE CELLS[1, 2]

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The cell transfer technique as initially described by Landsteiner and Chase (1) and developed by the Harrises (2) and others offers a unique opportunity for quantitating the antibody response made by a known population of cells. We have used this technique to determine the amount and kind of antibody produced as a result of the transfer of a given number of transferred rabbit lymph node or peritoneal exudate cells in response to a secondary stimulus by bovine serum albumin. The morphologic characteristics of these cells at time of transfer and throughout the secondary response have been correlated with the immunologic observations.

In the present studies the scheme of cell transfer was as follows: *1*) Donors were immunized by repeated intravenous and subcutaneous injections of bovine serum albumin (BSA) totaling 230 mg over a period of one month. *2*) Approximately 20 days after the last injection of antigen the donors received an intraperitoneal injection of 100 cc of highly viscous mineral oil. *3*) Three days after the injection of mineral oil the donors were sacrificed. The mesenteric and popliteal lymph nodes from all the donor animals were pooled as were the peritoneal exudates. Both pools were prepared as free cell suspensions, counted and transferred to recipient rabbits. *4*)

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[2] This is Reprint No. 59 of the Department of Pathology, University of Pittsburgh School of Medicine.

Recipient rabbits which had no previous exposure to BSA were in most instances x-radiated (400 r whole body) two days prior to receiving cells. Transferred cells were injected subcutaneously and intramuscularly into the ventral abdominal walls of the recipients so that the injection sites could later be removed for histologic study *5*). Following transfer of cells, BSA was injected intravenously into recipient rabbits.

In typical experiments approximately one-half billion lymph node or peritoneal exudate cells from previously immunized donor rabbits were transferred into the abdominal wall of an x-rayed recipient rabbit and stimulated by 1.5 mg BSA. One-half billion lymphoid cells represent approximately 0.5–1% of the total lymphoid tissue of a normal 2.5 kg rabbit (3). The distribution of cell types from each of these two sources was relatively consistent from one group of donors to another and in nine experiments averaged as follows: 1) lymph node cells—lymphocytes, 88%; macrophages and reticulo endothelial cells, 8%; plasma cells, 3%; miscellaneous, 1%; 2) Peritoneal exudate cells—macrophages, 71%; lymphocytes, 12%; polymorphonuclear cells, 13%; plasma cells, 1%; miscellaneous, 3%.

The macrophages of the peritoneal exudates are no doubt derived from a variety of sources including histiocytes and fibroblasts from the adjacent connective tissues, mesothelial lining cells of the peritoneal cavity and lymphocytes from blood or lymphoid nodules of the omentum.

Both the lymph node and peritoneal cells were capable of secondary immune responses following their transfer to x-rayed unresponsive hosts, and the responses by both were remarkably similar. The response of the peritoneal cells to BSA is in contrast to their reported inability to respond to bacterial antigens in cell transfer experiments (4). The time for elimination of circulating antigen in either type of transfer was approximately 5 days, which is comparable to the time of the anamnestic response in intact rabbits. The maximum concentration of circulating, precipitating antibody which was found 3–4 days after elimination of antigen, averaged 23.0 μg anti BSA N/ml serum for 31 recipients of peritoneal cell transfers and 20.4 μg for 35 recipients of lymph node cell transfers. At this time the total circulating antibody in the recipient was equivalent to approximately ¼ of the wet weight of the transferred lymphocytes and $\frac{1}{12}$ the wet weight of the transferred macrophages. When these values are converted to molecules of antibody/transferred cell, we find that in the case of both peritoneal and lymph node cells for every cell transferred somewhat more than 200 million molecules of anti-BSA were circulating in the recipient 8 or 9 days later (5).

Based on these observations, one can make certain estimates concerning the minimum antibody synthesis resulting from the transfer of a known number of cells. First, the total antibody synthesized during the first 8 days of the response is probably two or more times that present in the recipient on the 8th day, since considerable antibody combines with circulating antigen and is rapidly catabolized early in the response and additional antibody is lost by normal, non-immune catabolism prior to day 8. Next, it is obvious that all cells do not have an opportunity to participate in the immune response since many die within the first few days after transfer, as revealed by histologic observation. Since it is impossible to quantitate the proportion of transferred cells which are lost, this variable can best be minimized by basing estimates on the most successful transfers with the least necrosis of cells and the greatest circulating antibody. With both lymph node and peritoneal cells the recipients with the most successful transfers had antibody levels about 5 times the average figures given earlier. In these instances the transferred lymph node cells were responsible for the synthesis of more than two times their own wet weight in antibody within 8 days and the peritoneal cells were responsible for an amount of antibody approximately equal to their own wet weight. If these estimates of antibody synthesis are correct and if, as seems likely, the transferred cells are in large part responsible for antibody synthesis, it means that cells making a secondary antibody response are among the most active protein producers and secreters in the body. If the antibody response in intact animals is similar to that in transferred cells it would appear that a relatively small proportion of the total lymphoid tissue of an animal could be responsible for sizable antibody responses.

That the transferred donor cells alone are primarily responsible for this antibody synthesis seems likely for several reasons: *a*) The recipient rabbits were exposed to 400 r whole body x-radiation, which rendered them incapable of responding to bovine serum albumin. *b*) The

short induction period and prompt elimination of antigen observed in these experiments was consistent with a secondary response, as would be expected from the transferred cells. The observed response was 5–6 days earlier than would have occurred had the recipient rabbits been making a primary response to the antigen. *c*) The initial combining ratios of the antisera ranged from 8–16, values characteristic of a secondary response and significantly higher than is found in the serum in a primary response. *d*) The amount of antibody observed in the recipients was much more than is formed by normal rabbits during a primary response to 1.5 mg doses of BSA.

In ascribing the antibody response to the transferred cells alone, one possible reservation should be considered. That is, even though the x-rayed animals alone could not form antibodies, the transferred cells might supply a factor which would then enable the cells of the recipient animal to make a typical secondary response to an antigen to which they had not been exposed before. This, however, does not seem likely in view of the fact that in the cell transfer studies of Wager and Chase employing the diphtheria antitoxin system, transfer of previously stimulated cells containing antigen to normal recipients resulted in not only a secondary response on the part of the transferred cells but also a primary response on the part of the recipient (6), indicating that the ability to make a secondary response had not been imparted to the recipient. In addition, in our experiments the donor cells were effective in eliciting a response only if they are viable. No immune response was associated with the transfer of frozen-thawed, x-rayed, or heat-killed cells.

Since neither the lymph node cells nor the peritoneal exudate cells were of a single morphologic type, it is not certain which type or types of cells were responsible for antibody synthesis in either case. However, because the amount of antibody synthesized is so great, it is tempting to attribute its synthesis to the major cell type in each preparation, i.e. the lymphocytes for the lymph nodes and the macrophages for the peritoneal exudates. In an attempt to determine whether the lymphocytes which made up 10–15% of the peritoneal cells could alone have accounted for the response of the peritoneal exudates, it was shown that lymph node lymphocytes equal to or somewhat greater in number than the lymphocytes in the peritoneal exudate transfers were alone unable to transfer a detectable response. This strongly suggests a major role for the macrophages in the response of the peritoneal exudate cells. Conversely, peritoneal macrophages equal in number to the reticulo-endothelial macrophage elements in the lymph node preparations alone could not transfer a detectable response. Since there was relatively little multiplication of the transferred cells during the secondary response (less than 1 mitosis/1000 viable transferred lymph node cells as seen in histologic preparations from 1–5 days after transfer), it is unlikely that any of the rarer cell types increased greatly in number after transfer.

If the lymph node and peritoneal cell responses were caused by different cell types, it seemed possible that some immunochemical or biological differences between the responses might be found. However, to date all attempts to detect such differences have failed. The avidities of the antibodies from the two types of cell transfer as expressed by the dissociation constants have in several instances been determined by Dr. Richard Farr and found to be identical (7). The x-ray sensitivity of the antibody responses by lymph node and peritoneal cells also appear to be the same; 500 r given to either kind of cell *in vitro* prevents an immune response following transfer of the cells to x-radiated recipients. Finally, following antigenic stimulation there seems to be no consistent difference between the duration of antibody synthesis by these two different cell populations.

Since it has been repeatedly demonstrated that the antibody response is associated with morphologic alterations in the cells presumably synthesizing antibody (8), it is essential, in comparing morphologic and immunologic behavior of cells, to follow them throughout the response. In the present study, histologic observation revealed remarkable similarities in the development of the cells in both lymph node and peritoneal exudate transfer sites throughout the response, in apparent agreement with the similarities in their immunologic behavior. These histologic observations were made by sacrificing recipients at varying intervals after cell transfer and removing the transfer sites for study. Transfers of killed cells and of living cells which for various reasons did not make an immune response served as controls for comparison with transfers of cells which made detectable responses. The sequence of cellular changes in the transfer sites was as

follows: *1*) After one day the transferred cells could be found in the edematous injection sites with relatively little evidence of a host reaction. *2*) At two days there was death of a variable number of transferred cells and a granulocytic host response which was more marked with peritoneal cell transfers. *3*) From 3–6 days the number of recognizable lymphocytes or macrophages decreased and immature plasma cells developed throughout the injection sites, roughly in proportion to the amount of the antibody formed. The granulocytic response diminished and a giant cell and fibroblast response developed. *4*) From the 6th to the 8th or 9th day with both lymph node and peritoneal cell transfers mature plasma cells were prominent, while the giant cell and fibroblast host responses increased. The amount of antibody synthesized could be correlated with the number of plasma cells found in the transfer sites. Prediction of good or poor antibody response on the basis of histologic study alone was accurate in 46 of 54 transfer sites studied. *5*) After transfer of killed cells or living cells which for various reasons did not make an immune response, the recipient response developed much as described above, but few or no plasma cells were seen.

From these observations it would appear that both the lymph node and peritoneal cells are capable of transferring the ability to make a secondary antibody response during which large numbers of typical plasma cells appear in both types of transfer sites; and that the number of plasma cells which appears is correlated with the amount of antibody synthesized. It seems possible in these experiments that the plasma cells develop from the lymph node lymphocytes and peritoneal macrophages much as they do from the large primitive reticulo endothelial precursors in the lymph nodes and spleens of intact animals making immune responses or in organ cultures of lymphoid tissue synthesizing antibody *in vitro* (8). That lymphocytes and macrophages should, along with the primitive reticulo endothelial cells, be able to go down the path leading to antibody formation and pyroninophilia is in agreement with the belief of many histologists that there is a close relationship between the various morphologic entities in the lymphoid-reticulo endothelial system.

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MECHANISM OF THE SECONDARY ANTIBODY RESPONSE TO PROTEINS[1]

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For a number of years we have been investigating the sites and mechanisms of antibody formation, including especially the secondary or anamnestic response which was first described about 50 years ago by Solomonsen and Madsen (1). This reaction is manifested by a prompt, extensive and prolonged antibody response to a second injection of protein in contrast to the delayed, small and shorter-lived response to the first inoculation. It is remarkable that in certain cases antigenic restimulation months or even years after the initial injection can evoke this exaggerated response (2).

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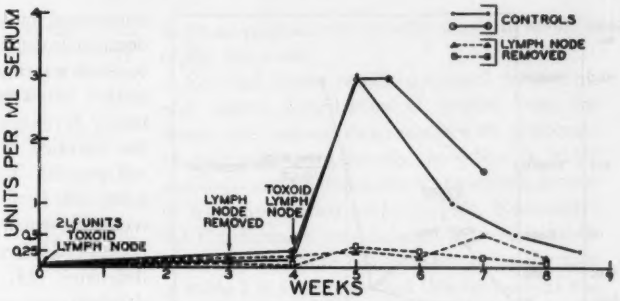

FIG. 1. Primary and secondary antitoxin response in control rabbits and rabbits after removal of popliteal lymph node. Antitoxin units as determined by skin test assay.

Some 10 theories (2–5) have been proposed to explain antibody formation. It has been clear for long, however, that to obtain a deeper understanding of this process it would be necessary to develop techniques for the study of the antibody-synthesizing apparatus apart from the many factors which influence antibody formation in the whole animal. Two such methods are the cell transfer phenomenon (6) and the *in vitro* production of antibody by organs of immunized animals (7, 8). These two techniques are being employed concurrently in an effort to develop a more comprehensive understanding of the nature of antibody synthesis.

Our initial aim was to localize the site of antibody production. Since spleen and lymph nodes had been implicated as loci of antibody formation against particulate antigens such as red cells and bacteria (9), these organs were examined to determine whether they participated in the antibody response to soluble proteins such as toxoids. Diphtheria toxoid was employed because a second injection of this protein yielded more antibody per milligram of antigen than other proteins. These studies were expedited by use of a modification (10) of the sensitive hemagglutination method of Boyden (11).

The initial experiments (12, 13) employed the classical extirpation method in which the effect of removal of a particular organ was assessed in relation to the antibody response of the whole animal to toxoid. Figure 1 illustrates a typical experiment. The control animals manifested a typical booster response to the second intranodal injection of alum-precipitated diphtheria toxoid. On the other hand, when the lymph node was removed before the second injection, the rabbit developed a feeble antibody response to that injection. Similarly, when antigen was injected intravenously or into the spleen itself, removal of the spleen resulted in diminished antibody production. Thus, the lymph node was involved in the booster reaction to toxoid after local antigenic stimulation, whereas the spleen took part upon intravenous injection. Other investigators (9, 14) have reached the same conclusions from different types of evidence.

We next (12, 13, 15) sought experimental evidence for the direct participation of lymph node and spleen cells in antibody production. It was, for instance, conceivable that these organs were not themselves responsible for the final synthesis of antibody, but were producing one component of a system involved in the over-all process and this component was transferred to another organ where antibody actually was made.

As shown in figure 2, 20 Lf units of alum-precipitated diphtheria toxoid were inoculated into the hind foot pad of a rabbit twice with an interval of 3 wk. between injections. Three days after the second injection the popliteal lymph nodes were removed, cells prepared and injected into normal rabbits. In another series splenic cells were transferred after two intravenous injections of the donor. The animal which receives the cells is called the recipient or host. As illustrated in figure 3, cell transplantation caused the appearance of increasing amounts of antitoxin in the blood of the recipient during the next 5 days. The extent and speed of antibody production in the host should be contrasted with the small amount of antitoxin which appeared only after 14–21 days in rabbits injected for the first time with toxoid. Moreover, only 2 out of 100 recipients developed a delayed primary antitoxic response to toxoid contained in the donor cells (16). Even more impressive was the fact that only 2 out of 50 of the same series of 100 recipients manifested a secondary antitoxin response when the same dose of toxoid was injected into them 3 wk. after the cell transfer (16).

The cell transfer phenomenon also has been elicited following the use of tetanus toxoid and

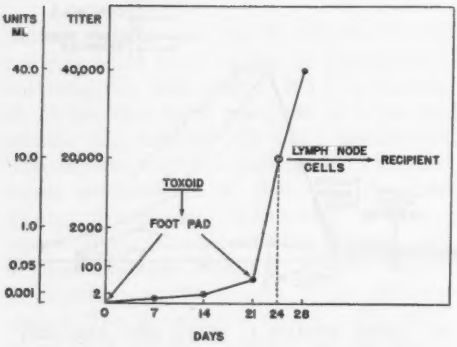

FIG. 2. Preparation of donor for transfer of lymph node cells.

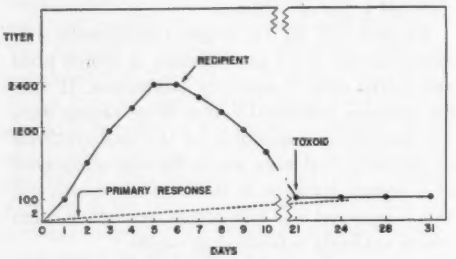

FIG. 3. Response of recipient to cell transfer from immunized rabbit.

bovine gamma globulin in the rabbit and diphtheria toxoid in the rat.

The data presented to this point suggest that the transferred lymph node and spleen cells participate directly in antibody production in recipient animals. However, before further study could be made of these cells as the actual sites of antibody production, it was imperative to show more conclusively that synthesis of new antibody was occurring rather than conversion of a precursor to antibody.

Several plausible explanations of the cell transfer data have been offered: *1*) an active immunization of the recipient by antigen that is contained within the transferred cells; *2*) the transfer to the recipient of preformed intracellular antibody or a conversion in the recipient of an intracellular protein or non-protein precursor to antibody; *3*) actual antibody synthesis by the transplanted cells.

Active immunization of recipient was unlikely because of the rapidity and extent of antibody production in the host contrasted with the slow and small antibody response of an animal actively immunized once with toxoid. In addition the donor cells usually did not contain enough toxoid to elicit a primary antibody response upon injection into a host. Nor did the recipient commonly develop a secondary antitoxic reaction to the injection of toxoid several weeks after the cell transfer. Moreover, antibody was produced when cells from immunized donors were given to recipients which could not be actively immunized because of x-irradiation (17) or pantothenic acid deficiency (18). The observations in vitamin-deficient rats are particularly reminiscent of findings of the Harrises on x-irradiated rabbits (17) in that more antibody was formed in vitamin-deficient than in normal rats (18). We believe these results are explicable on the basis of better survival of the transferred cells and hence more prolonged antibody formation since the deficient recipients are unable to develop a normal immune response against the transplanted cells. The experiments just mentioned were conducted with A. E. Axelrod who first showed (19) that pantothenic acid-deficient rats did not produce antibodies against cellular antigens or diphtheria toxoid.

Thus these experiments, like the previous ones, provide indirect evidence pointing to the transferred cells as the site of antibody synthesis.

Transfer of antigen, antibody or antibody precursor material might not be expected to require intact cells; however, it has been found (6, 12–15) that treatment of donor cells by heating to 40° C for 15 min. or freezing and thawing abolished their antibody-forming capacity. Moreover, transplantation of cells from one species to a heterologous species should convey either intracellular antigen or antibody to the recipient. However, numerous transfers of cells from immunized rabbits to normal rats did not lead to antibody production. Others have made similar unsuccessful attempts involving various combinations of heterologous species (14, 20).

Not enough antibody has been detected in the donor cells to account for the amount of antibody found in the host (12–15). Again, however, these findings do not exclude the possibility that the cells contained either antibody in unextractable form or a serologically-inactive precursor of antibody.

Some evidence of synthesis of new antibody in the host has been presented. Chase (21) showed that the avidity of the antitoxin arising in the recipient animals reflects the quality of

TABLE 1. COMPARISON OF TOTAL PROTEIN AND GAMMA GLOBULIN CONTENT OF TRANSPLANTED LYMPHOID CELLS WITH THE AMOUNT OF SPECIFIC ANTIBODY GLOBULIN IN RECIPIENTS

Exper.	Transplanted Cells*		Total Antibody, globulin†
	protein	γ globulin	
	μg	μg	μg
1	35	2	40,000
2	45	4	15,000
3	—	270	9,000

Donors: Diphtheria toxoid injected into rear foot pads on day 1 and 30. Lymph nodes removed on day 33 for transfer.

* In experiments 1 and 2 the cells from 1 lymph node were transplanted, whereas in experiment 3 the cells from 5 nodes were injected.

† At height of antibody response in recipients.

the antitoxin being produced by the donor at the time of sacrifice; whereas, if host antitoxin represented a primary response to toxoid or transfer of an 'intermediate' type of antigen, it should have the avidity characteristic of the primary antibody response. However, the avidity data also can be explained by the presence of bound antibody or precursor in the donor cells.

The most direct way to exclude the transfer of bound antibody or precursor was to compare the total protein and gamma globulin content of the donor cells with the amount of antibody which appeared in the host at the height of its response (22). Protein was determined by the Lowry method (23) applied to trichloracetic acid precipitates of cell extracts, and both gamma globulin and antibody were measured by the quantitative precipitin method (24). Table 1 shows that there is a tremendous discrepancy between the protein and gamma globulin content[2] of the donor cells and the total antibody of the recipient. Thus there is not nearly enough protein in these cells to account for the antitoxin in the recipient (22, 25). Recently Roberts and Dixon (26) reached a similar conclusion by comparing the amount of antibody in the host with the calculated weight of the lymph node cells which were transferred. However, the discrepancy between the amounts of available cell material and antibody produced in the host did not appear from their calculations to be as great as was actually found by analysis of the donor cells.

Although these results appeared to exclude any sizable contribution of protein from the donor cells, isotope experiments were performed to determine more directly the extent to which antibody was produced from preexistent protein or from free amino acids (22, 25). Radioactive S_{35} L-methionine was injected intraperitoneally into recipients after cell transfer. From the data in table 2 it is obvious that this amino acid was incorporated into the antibody produced in the recipient. It is significant that the specific radioactivity of the methionine in this antibody closely approximated the specific activity of the methionine in antibody produced by actively immunized animals which had been given equal amounts of radioactive methionine (22, 25).

It was then found that when S_{35} labeled normal gamma globulin was injected into recipients after cell transfer the antibody which subse-

TABLE 2. INCORPORATION OF S_{35} L-METHIONINE INTO ANTIBODY PRODUCED IN RECIPIENT ANIMALS

Exper.	Animal	Antigen	Cells	Radioactivity injected*	Recipient's Serum	
					Preparation	Activity c/m per
				μc		
1	rat	toxoid	spleen	350	Serum + T + AT	1282 mg ppt
					Serum + EA + Anti EA	262 mg ppt
2	rabbit	BGG	spleen	650	Serum + BCG	961 mg antibody
					Serum + EA + Anti EA	56 mg ppt
3	rabbit	toxoid	lymph node	375	Serum + T	58 mg antibody
					Serum + BGG + Anti BGG	11 mg ppt

Donors: All were injected on days 1 and 30 and transplants made on day 33. In experiment 1 toxoid was injected intraperitoneally, in experiment 3 into the rear foot pads. In experiment 2 injections were made intravenously.

Transfers: In experiment 1, S_{35} methionine was injected with cells and animal bled out after 2 days. In other experiments the amino acid was injected at or near peak antibody response and the animals bled out 12 hr. later.

Abbreviations: T = diphtheria toxoid; AT = rat diphtheria antitoxin; EA = egg albumin; Anti-EA = rabbit antibody to EA; BGG = bovine gamma globulin; Anti-BGG = rabbit antibody to BGG; μ = microcuries.

* 16.3 millicuries/gram.

[2] It is likely that the gamma globulin detected represented contamination of the extract with plasma gamma globulin and was not actually associated with the intracellular environment.

quently was formed was not radioactive. Similar findings have previously been made in actively immunized animals by Gros *et al.* (27). However, since antibody production undoubtedly is a cellular process it is possible that circulating gamma globulin did not readily gain entrance into antibody-synthesizing cells and consequently did not participate in antibody synthesis.

In another series of experiments (22, 25) it was observed that the transplantation of donor cells whose protein was highly labeled with S_{35}-L-methionine did not lead to labeling of the antibody in the recipient. These results again suggest that neither antibody nor a protein precursor such as gamma globulin is present in the transferred cells and that antibody is formed from other raw material in the recipient animal. Anker and Green (28) from isotope experiments in the whole animal previously had also concluded that gamma globulin was not a precursor of antibody globulin and antibody was probably derived from free amino acids.

After our experiments were completed, Taliaferro and Talmage (29) reported similar results from similar transfer experiments in which recipients were given either radioactive amino acids or labeled donor cells.

The foregoing data favor the concept that cell transplantation leads to synthesis of new antibody in the recipient. Incorporation of amino acids occurs in association with the appearance of this antibody. Normal gamma globulin is not a precursor of antibody. There is not enough preformed protein in the transplanted cells to account for the amount of antibody which is synthesized. Therefore, most of the protein which is present in the cells is not converted to antibody. It is, nevertheless, possible that amino acids are converted to a protein of the structural complexity of antibody itself, but that very little of this precursor material is accumulated in the cells.

The transplanted cells may remain functional for at least 3 days in the recipient because injection of antigen into the host 3 days after cell transfer resulted in a booster response (16, 26). Within the next 4 days these cells slowly lose their capacity for the anamnestic reaction (16). Thus there appears to be a good correlation between the viability of the transplanted cells and continued antibody formation in the recipient. Moreover, the transferred cells possess considerable autonomy as indicated by their ability to function in recipients which are unable to make antibody because of x-irradiation (17) or pantothenic acid deficiency (18). However, further study is required to determine whether the transplanted cells make antibody entirely by themselves or whether their host appropriates and utilizes those elements of the transferred cells essential to the process.

Transfer of spleen or lymph node fragments from immunized animals apparently results in antibody synthesis by cells within the fragments. The extensive studies of Hale and Stoner (30) and Oakley and his associates (31) support this conclusion. Further evidence is provided by the finding in our laboratory (16) that, when splenic fragments are transplanted from immunized rabbits to the anterior chamber of one eye of the rabbit, antitoxin can be detected in the fluid from this eye at a time when fluid from the other eye did not contain antibody.

Depending on the route of injection and the dose of antigen employed, these experiments resulted in antibody production by different organs (13, 16). When antigen was injected intravenously, the spleen was active; when toxoid was inoculated into various sites the regional lymph nodes were stimulated. However, difficulty was experienced in inducing antibody formation in the mesenteric nodes. This difficulty was only partially overcome by injection of large amounts of antigen intraperitoneally or small amounts directly into these nodules. Similar data have been presented by others (14, 26).

Our *in vitro* studies will be mentioned because they substantiate some of the conclusions derived from the cell transfer studies and also permit experiments of a type not possible by the transplantation technique. These experiments were based on previous observations (2, 7, 8) that lymphoid tissues from immunized animals could produce antibody *in vitro*. With the collaboration of Mr. Benjamin Wolf, a suitable *in vitro* antibody-producing system was developed (22, 32, 33). Lymph nodes were removed from immunized rabbits 3 days after the booster injection of diphtheria toxoid and cut into fragments which were incubated for 20 hr. at 37° C in Fischer's V-614 medium containing 20% rabbit serum. At the end of incubation there was 5–100 times more antitoxin in the medium than could be extracted from unincubated fragments. *In vitro* antibody production was sharply limited to tissues removed 3 days after the booster injection (33). Apparently the tissues had to remain in the donor for 3 days after injection of antigen

so as to undergo some physiological process essential to antibody production which could not occur *in vitro*. In view of recent studies (8, 34) this process may consist of the development of immature plasma cells.

Energy apparently is required for *in vitro* antibody formation since inhibitors of energy-yielding reactions such as potassium cyanide and 2,4 dinitrophenol inhibit the process completely (22, 32, 35).

Antibody synthesis also was inhibited when amino acid analogs such as *p*-fluoro phenylalanine or the gamma ethyl amide of glutamic acid were added to the medium containing lymph node fragments (33). This inhibition was reversed by addition of the corresponding natural amino acids to the medium, which suggests that the analogs competitively inhibit the utilization of the natural amino acids for antibody formation.

In confirmation of the earlier observations of Ranney and London (36) and Keston and Dreyfus (37), radioactive S_{35} L-methionine was incorporated into the antibody produced *in vitro* (22, 33). However, it is possible that this incorporation represents exchange (38) of radioactive amino acid with the corresponding nonradioactive amino acid in preformed antibody. Further study is needed to clarify the relationship between *in vitro* net synthesis of antibody and incorporation of amino acids into this protein.

Recent experiments (33) have provided strong evidence that the antibody detected *in vitro* represents new antibody formed predominantly from free amino acids. Lymph node proteins were labeled by injecting S_{35} yeast protein hydrolysate into the immunized donor animal. When these labeled lymph node fragments were cultured, the antibody which appeared in the medium was not radioactive. Thus this antibody could not have been derived from the proteins of the node, but came from free amino acids.

As a rather free speculation, a conceptual scheme may be envisioned which accommodates the available observations and also serves as a working hypothesis to direct future studies. It is assumed that antibody synthesis requires a specific template other than antigen but that antigen serves both to induce the synthesis of the template and also to trigger the anamnestic response in cells previously exposed to antigen. These assumptions replace the previous ones (2, 3, 39) that the antigen directly impresses its configuration either upon the existing normal gamma globulin or upon a polypeptide precursor of this globulin. In unmodified form the previous schemes appear incapable of explaining the well-known finding that antigenic restimulation months or even years after the initial injection can evoke an anamnestic reaction (2), unless it is assumed that antigens can persist for this length of time. Such persistence seems quite unlikely unless the antigen is a living virus that can undergo multiplication. However, although it is more plausible to assume that antigen acts as the inducer of the template rather than as the template itself, it is conceivable—although improbable—that a breakdown product of the antigen may persist as the actual inducer, but that this metabolite may be unable to detonate the anamnestic reaction in cells already induced by the primary exposure to antigen.

Figure 4 presents a thumbnail sketch of the primary antibody response. Following injection the antigen enters into appropriate cells and evokes some specific alteration in their physiology. By analogy with recent studies on other protein-synthesizing systems (40) this change may consist of the development in the cytoplasm, possibly within the microsomes, of a template for the synthesis of specific antibody. The specific structure containing the template is then replicated during mitotic division of the cells so that during the several weeks after introduction of antigen an increasing number of cells in a lymph node, for example, acquire the potentiality of synthesizing antibody.[3] During mitosis, however, antigen is diluted and degraded intracellularly so that several weeks after the primary injection relatively few cells possess enough antigen to stimulate appreciable antibody synthesis; as a result there is only a slow rise in serum antibody. This scheme pictures the response obtained with diphtheria and tetanus toxoids. On the other hand, proteins such as bovine gamma globulin may rapidly induce production of the template and hence rapid production of appreciable amounts of antibody, after the first injection, but only a relatively small rise in antibody following the second injection.

Figure 5 presents a schematic view of the secondary response to the reintroduction of antigen at this time. In cells which do not contain

[3] Whether the template, once induced by antigen, can replicate in the absence of antigen is a very fundamental question which cannot be answered by the available data.

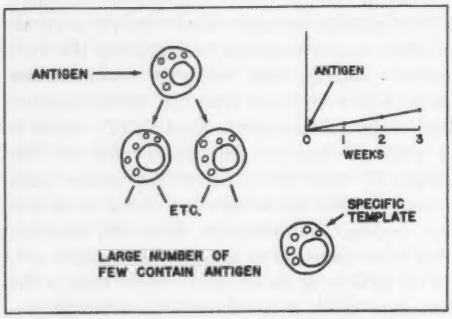

FIG. 4. Conceptual scheme of cellular response to primary injection of proteins. Spheres within cytoplasm of cells represent the templates. Insert graph depicts the actual antibody response.

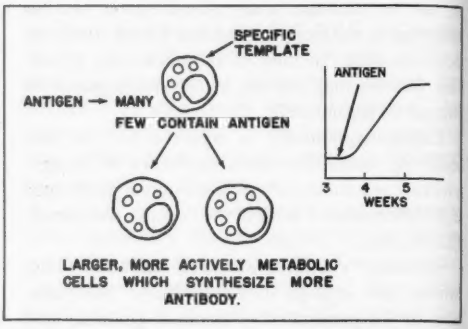

FIG. 5. Conceptual scheme of cellular response to secondary injection of proteins. Spheres within cytoplasm of cells represent templates. Insert graph shows the actual antibody response.

specific template material, antigen could produce the same effects as followed the primary inoculation. On the other hand, cells which contain the specific template, might react in a strikingly different way to antigenic restimulation. The cells might differentiate into immature plasma cells, multiply more rapidly, and correlated with this general increase in what might be called their basal metabolic rate, might synthesize more antibody.

The picture apparently is different when bacterial polysaccharides are employed (41). These substances may gain entrance to the cells more readily and are probably less readily destroyed enzymatically than proteins. All of the cells potentially capable of forming antibody probably are doing so because they always contain antigen. Consequently, antibody production is maintained at a rather constant level for a long time and the reinoculation of antigen will not elicit a booster response.

This general scheme is far from original. The majority of its elements can be found in the writings of Burnet (2), Haurowitz (3), Coons (34), Heidelberger (41) and Stalleybrass (4) to mention just a few contributors. It is encouraging that there is a good deal of evidence which is at least in general accord with the postulated events. For instance, Coons (34) recently has presented visual evidence for the presence of larger numbers of antibody-containing cells in the lymph node after the secondary than after primary antigenic stimulation. In our experiments (13, 15, 16) the entity associated with antibody synthesis apparently was not transferred from the donor cells to those of the recipient because the recipient usually did not respond to a booster injection of toxoid 3 wk. after the transplantation. It is, therefore, quite likely that this entity is passed only from mother to daughter cells during mitosis and does not pass through the cell membranes from donor cells to cells of the recipient.

Recently in collaboration with Dr. Helen Harrington (42) we have obtained preliminary evidence of a general increase in the metabolism of the lymph node during the anamnestic reaction. Nodes removed 3 days after secondary antigenic stimulation of rabbits showed greater *in vitro* incorporation of radioactive P_{32} into the trichloracetic acid-soluble fraction, phospholipids, desoxyribonucleic and ribonucleic acid than nodes from normal animals. It has not been determined whether, in view of the recent studies of Coons (34), this increase in metabolism was associated with the development of a large number of immature plasma cells in the lymph node.

The analytical and isotope data presented (22, 25–29, 33) effectively rule out the release of stored antibody or a precursor as the basis of the anamnestic reaction. However, as mentioned previously, the evidence is insufficient to exclude the presence in the cell of small amounts of a rapidly-turning-over serologically-inactive precursor of the structural complexity of antibody itself. Figure 6 presents an over-all view of our present concept of antibody synthesis based on experiments from this and other laboratories. This concept is contrasted with the previous notion (2, 3, 39) of the conversion of normal gamma globulin or a polypeptide precursor to antibody under the direct influence of antigen.

PRESENT SCHEME

Amino acids → Antigen-induced template → Antibody γ Globulin
Amino acids → Distinct template → Normal γ Globulin

PREVIOUS SCHEMES

Amino acids → Polypeptide precursor or Normal γ Globulin → Antigen as template → Antibody γ Globulin

FIG. 6. Previous and present concepts of mechanism of antibody synthesis from amino acids.

Of course, these concepts are only provisional. Future work may relegate many of them to the wastebasket of history. Nevertheless, they are at least in broad agreement with the conclusions of recent genetic and biochemical studies of protein synthesis in bacterial and mammalian tissues (43, 44). The analogy between the secondary antibody response and the process of adaptive enzyme formation in bacteria is especially striking (44). In both cases the presence of a new and foreign substance, a substrate in enzymatic adaptation, an antigen in the case of antibody synthesis, evokes a specific alteration in the synthetic capacity of the special cells involved. In both instances a new protein is synthesized from free amino acids rather than from a polypeptide or protein precursor (44).

Aldous Huxley has said that the aim of the scientist is 'to put salt on the tail of the Absolute.' I do not know whether the tail of the antibody problem has been reached. However, with so many people wielding salt shakers, and in view of the rapid advances being made in the understanding of many related phenomena, it may be anticipated that in the next few years we shall acquire a much better comprehension of the fundamental mechanisms of antibody formation.

The author wishes to acknowledge the technical assistance in the course of these studies of Anita C. Jarchow, Bradley Brownlow and Anne D. Gardner, and the constructive criticism of his colleague, Dr. Charles Yanofsky.

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CORRECTIONS AND ADDITIONS TO MARCH ISSUE

Page 22. CHIEN (intr. by GREGERSEN). *Next to last sentence should read:* "With increasing blood-losses, the degree of hemodilution increased, and the sympathetic activity became more effective."

Page 74. KONIGSMARK AND FRENCH (intr. by SONNENSCHEIN). *Line 7:* delete "is" and substitute "are."

Page 89. MONKHOUSE AND MACKNESON. *Line 12 should read:* "The following levels expressed in units per gram of tissue . . ."

Page 104. RAYPORT (intr. by JASPER). *Line 16:* Delete chemical formula and substitute "ferric ammonium citrate."

Page 110. RUDOLPH AND OLSEN. *Second column, line 9* should read: "glucose-6-C^{14} was markedly decreased (85%)."

Page 139. YATES AND URQUHART (intr. by LANDIS). *Line 2:* "coritical" should read "cortical"; *line 4:* "stimulated" should read "simulated"; *line 6:* "constructed" should read "constricted"; *line 11*, "rate" should read "rare"; *line 22:* "ty" should read "by."

Page 205. KLINGMAN AND HANDLER. *Second column, lines 2 and 3* should read: "of the phosphate-activated glutaminase of kidney have not been established. . . ."

Page 232. PEIFER AND LUNDBERG. *Title should read:* "Influence of specific fatty acids on the development of arterial lesions in miniature pigs"; *line 21:* substitute "slight abnormality" for "a small amount of plaque formation"; *line 24:* delete "surface of the . . ."; *line 27 through end*, delete and substitute: "plays a predominant role in the development of the arterial lesions. The lesions appear to be different from those of human atherosclerosis."

Pages 232 and 233. PETERSON AND BEATTY. *Top of page 233, second half of abstract* should read "diabetics showed elevated B-fractions. Total serum proteins were lower in alloxan diabetics (-0.95 g % $P < .005$) and there was an increase of 0.29 g % ($P < .005$) in β-globulin, no change in α_2-globulin and a decrease in α_1-globulin ($P < .005$). Seven pancreatectomized rats with high fasting blood sugars showed a decrease of 8% in serum albumin ($P < .01$), a decrease of 2.8% in α_1-globulin ($P < .05$) and an increase of 6.7% in β-globulin ($P < .005$) compared with 17 controls. There was no change in total serum protein following pancreatectomy ($P > .10$). Six rats, deprived of pancreatic exocrine secretion, showed no change in % serum albumin, α_2 or β-globulins or total serum protein, and a decrease of 2.4% in α_1-globulin ($P < .05$). Changes in % albumin and β-globulin are similar to those reported in diabetic humans and do not agree with the changes reported in male alloxan diabetic rats (Nichols *et al. Diabetes* 5: 468, 1956. (Aided by PHS grants A-213 and A-1157, and the Glidden Co.)"

Page 275. YARD, WEATHERBY AND MCKENNIS, JR. In title and throughout text, "α-glutamylhydrazide" should read "γ-glutamylhydrazide." Author's name, "Watherby" should read "Weatherby."

Page 281. BECK AND RIECK. *Line 18:* delete last two sentences and substitute, "However, mice which had been adrenalectomized and were therefore presumably incable of releasing endogenous epinephrine, exhibited a smaller trauma induced decrease in liver NPSH than did either control or sham operated mice. Further investigation of this problem is in progress."

Page 360. HOFFBAUER, BARNUM AND ZAKI. *Line 4* should read "tion (hypertrophic cirrhosis); 4) irregular periportal regeneration (atrophic nodular cirrhosis) . . ."

Page 367. NELSON AND DAVIDOW. *Line 7:* "4, 2, 2, and 0" should read "4, 2, 0, and 0"; *line 21:* "(totals 50 vs. 39)" should read "(totals 49 vs. 38)".

Page 409. CHENG AND HAUROWITZ. *Third and fourth sentences* should read: "Serum samples were taken shortly before and 1 and 2 days after injecting OA-II. They were inactivated at 55° and found to be free of precipitating antibody."

didates have notified us that they have accepted positions. The larger number of interviews and the larger proportion of candidates receiving interviews all seem a result of the larger number of employers using the interview service and the increased number of available positions they represent.

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National Library of Medicine

The National Library of Medicine is to be located on the grounds of the Public Health Service's National Institutes of Health in Bethesda, Maryland. This choice selection of a site for the new Library building was based on many factors, among which was its proximity to two large medical centers.

International Conference

An International Conference on Scientific Information, sponsored by the National Science Foundation, the National Academy of Sciences and the American Documentation Institute, will be held in Washington, D. C., in November 1958. The Conference will be concerned with a critical examination of problems of scientific information, emphasizing in particular those of storage and retrospective search of information. Also emphasized will be the exchange and critical evaluation of information regarding the results of research on storing and retrieval. Working scientists and documentalists are encouraged to submit proposals for papers. Worthwhile papers will be accepted for inclusion on the program whether or not the author is able to attend the Conference. Any person desiring to become a participant in the Conference is urged to send the title and a topic outline of the paper or study he proposes to the Secretariat of the Conference. If the title and a general outline are acceptable for one of the areas, submission of the full paper will be invited. Further information can be obtained by writing: International Conference on Scientific Information, National Academy of Sciences, 2101 Constitution Avenue, Washington 25, D. C.

World Congress

The American Gastroenterological Association will be host to the World Congress of Gastroenterologic Societies in May 1958. The Congress will convene at the Sheraton-Park Hotel, Washington, D. C., May 25–31. Guest speakers from many nations will participate. Commercial and scientific exhibits will be held in conjunction with the Congress.

Science Teaching Improvement Program

In order to meet more effectively the goals of the Science Teaching Improvement Program, to increase the number of well-qualified science and mathematics teachers at the secondary school level, a grant from the General Electric Educational and Charitable Fund has enabled the American Association for the Advancement of Science to establish a plan for regional consultants to serve colleges and universities. Twenty consultants in science and mathematics are available to visit colleges and universities in their areas upon invitation. Interested institutions are urged to utilize the services of these consultants with a view to solving problems of science and mathematics teacher education. For a list of the consultants, write to Dr. I. E. Wallen, Assistant Director, American Association for the Advancement of Science, 1515 Massachusetts Avenue, N.W., Washington 5, D. C.

Institute of Radio Engineers

The Professional Group on Medical Electronics

the Institute of Radio Engineers, has announced a new 'affiliate' plan in which physicians, biologists, physicists and biophysicists are invited to participate. The 'affiliate' will pay only a reduced PGME fee. He will receive notices of all local and national PGME meetings, as well as newsletters and the official group publication. The PGME aims to bring together doctors, electronic engineers, biologists and biophysicists to discuss the use of electronic techniques in problems of medicine and biology. For further information write to Mr. L. G. Cumming, Technical Secretary, Institute of Radio Engineers, Inc., 1 East 79th Street, New York 21, New York.

Honors

Dr. George R. Cowgill, Professor of Nutrition at Yale University and Editor-in-Chief of the JOURNAL OF NUTRITION, is the recipient of the American Institute of Nutrition's Osborne and Mendel Award for 1957, in recognition of Dr. Cowgill's fundamental research contributions to the knowledge of B-vitamins and protein nutrition. The award, consisting of $1,000 and a scroll, was made at the annual meeting of the Federation in Chicago, April 17. Dr. Cowgill is Past Chairman of the Federation Board.

Dr. William Mansfield Clark, Professor Emeritus, Department of Biological Chemistry, Johns Hopkins University School of Medicine, has been selected as the recipient of the $5000 Passano Foundation Award for 1957. The award is being made to Dr. Clark in recognition of his long and distinguished career as investigator and teacher, and for his demonstration over the years of the importance of physical methods notably in the control of pH and of oxidation-reduction to the study of life processes. The Passano Foundation is sustained by annual contributions from the Williams & Wilkins Company, publishers of medical and scientific books.

The John J. Abel Award, sponsored by the Eli Lilly Company, was presented on April 17 to Dr. Dixon M. Woodbury at the annual banquet of the American Society for Pharmacology and Experimental Therapeutics in Chicago. The $1,000 award, given each year since 1947 for outstanding research in pharmacology, was received by Dr. Woodbury for his paper on chemical changes in brain fluid producing excitability and convulsions.

New Books

ALCOHOLISM, Basic Aspects and Treatment. Harold E. Himwich, Editor. Washington, D. C.: American Association for the Advancement of Science, 1957. 212 pp. $5.75 ($5.00 to AAAS members)

ANNUAL REVIEW OF MEDICINE, VOL. 8. Palo Alto, Calif.: Annual Reviews, Inc., 1957. 530 pp. $7.00; $7.50 foreign

ANNUAL REVIEW OF PHYSIOLOGY, VOL. 19. Palo Alto, Calif.: Annual Reviews, Inc., 1957. 639 pp. $7.00; $7.50 foreign

CLINICAL TOXICOLOGY OF COMMERCIAL PRODUCTS —ACUTE POISONING (HOME AND FARM). Prepared at the Univ. of Rochester under the direction of Prof. Harold Hodge, Dr. Robert Gosselin and Mrs. Marion Gleason. Baltimore: Williams & Wilkins Co. 1957 $16.00

CYTOLOGY AND CYTOGENETICS. Carl P. Swanson. Englewood Cliffs, N. J.: Prentice-Hall, Inc., 1957. 596 pp. $13.35

ESSENTIALS OF PHYSIOLOGY, Bainbridge and Menzies, 10th Ed. Edited by H. Hartridge and J. L. D'Silva. New York: Longmans, Green and Co., 1957. 506 pp. $7.00

EXPECTANT MOTHERHOOD. Nicholson J. Eastman. Boston: Little, Brown & Co., 1957. 198 pp. $1.75

GUIDE TO MEDICAL WRITING. Henry A. Davidson. New York: The Ronald Press, 1957. 338 pp. $5.00

INSTRUMENTATION IN BIO-MEDICAL RESEARCH. Biology Council. Washington: National Academy of Sciences-National Research Council, 1957. 14 pp. $0.75

INTERNATIONAL CONGRESS OF GASTROENTEROLOGY, London, July 18–21, 1956. Basel-New York: S. Karger, 1957. 762 pp.

LIVER, BILIARY TRACT AND PANCREAS. Frank H. Netter. DIGESTIVE SYSTEM. CIBA COLLECTION OF MEDICAL ILLUSTRATIONS, PT. III, VOL. 3. 1957. 165 pp. $10.50

MEDICAL EFFECTS OF THE ATOMIC BOMB IN JAPAN, DIV. VIII, VOL. 8, National Nuclear Energy Series. Edited by Ashley W. Oughterson, Shields Warren. New York: McGraw Hill Book Co., Inc., 1956. 477 pp. $8.00

THE ADRENAL CORTEX. I. Chester Jones. New York: Cambridge University Press, 1957. 315 pp. $7.00

THE MECHANIZATION OF DATA RETRIEVAL (Studies in Coordinate Indexing, Vol. 4). Washington, D. C.: Documentation Inc., 1957. $5.00

THE METABOLIC RESPONSE TO NEONATAL SURGERY. P. P. Rickham. Cambridge, Mass.: Harvard University Press, 1957. 93 pp. $5.00

THE MICROBIAL WORLD. Stanier, Dondoroff and Adelberg. New York: Prentice-Hall, Inc., 1957. 682 pp. $8.00

THE RIDDLE OF STUTTERING. C. S. Bluemel. Danville, Ill.: The Interstate Publishing Co., 1957. 142 pp. Casebound, $3.50; Paper bound, $1.50. Four 12″ longplay Speech Therapy recordings @ $3.00 ea.

THERMODYNAMIC PROPERTIES OF THE ELEMENTS, Advance in Chemistry Series. Stull and Sinke. Edited by the Staff of Industrial and Engineering Chemistry. Washington, D. C.: American Chemical Society, 1956. 234 pp. $5.00

Federation of American Societies for Experimental Biology

Exhibit A. Balance Sheet—December 31, 1956

	Cost	Reserves for Depreciation	
ASSETS:			
Cash in banks			$ 6,045
Accounts receivable			20,324
Building and loan deposits			20,687
Supplies inventory			3,024
Prepaid expenses and deposits			8,236
Fixed Assets:			
Land	$ 25,770	$ -0-	25,770
Buildings and improvements	108,109	7,263	100,846
Annual meeting equipment	5,690	2,430	3,260
Other furniture and equipment	22,851	6,499	16,352
Trust fund investments at cost (market value $25,800)			16,605
Total Assets			$221,149
DEDUCT—LIABILITIES:			
Accounts payable and accrued expenses			$ 16,386
Note payable to the Riggs National Bank—4½% (secured by pledge of land and building; payable $2,250 semi-annually)			51,000
Deferred income			51,433
Total Liabilities			$118,819
NET ASSETS, REPRESENTING FUND CAPITAL (PER EXHIBIT B)			$102,330

Exhibit B. Statement of Income and Expenses and Fund Capital for Year Ended December 31, 1956

INCOME:	
FEDERATION PROCEEDINGS:	
Assessments on member societies	$ 18,152
Subscriptions, advertising, reprints, etc.	61,313
Annual meeting	74,236
Rental income	5,212
Grants and Government contracts	15,217
Transfer from International Physiological Congress Trust Fund	22,705
Dividends and other income	3,155
Total Income	$199,990
EXPENSES:	
Salaries, wages, Social Security and group insurance	$ 84,313
Printing and engraving	37,836
Advertising and exhibit commissions	17,409
Projection and public address services	5,637
Auditing and other services	5,612
Travel awards to XXth International Physiological Congress	22,000
Rentals of meeting rooms, exhibit halls and equipment	6,159
Supplies and duplicating	4,457
Communications, shipping and travel	9,413
Repairs and maintenance	1,009
Depreciation of buildings and equipment	6,226
Real estate taxes	2,039
Utilities	1,463
Interest expense	2,426
Other expenses and supplies	3,622
	$209,611
LESS: Portion of business services paid by American Physiological Society	−24,713
Total Expenses	$184,898
EXCESS OF INCOME OVER EXPENSES	$ 15,091
FUND CAPITAL:	
Balance, December 31, 1955	101,778
Add—Gain realized on sale of securities	8,166
Deduct—Transfer to the XXth International Physiological Congress Fund	−22,705
Balance, December 31, 1956	$102,330

FEDERATION PROCEEDINGS *Supplement no. 1*
July 1957

Interpretation of Data Obtained in Studies With Isotope-Labeled Proteins of Biological Significance

PANEL DISCUSSION SPONSORED BY THE METABOLISM AND NUTRITION STUDY SECTION, DIVISION OF RESEARCH GRANTS, NATIONAL INSTITUTES OF HEALTH, UNITED STATES PUBLIC HEALTH SERVICE, ATLANTIC CITY, NEW JERSEY, APRIL 19, 1956

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FEDERATION *of* AMERICAN SOCIETIES *for* EXPERIMENTAL BIOLOGY

The Metabolism and Nutrition Study Section

The Metabolism and Nutrition Study Section is one of the scientific advisory groups of the Division of Research Grants of the National Institutes of Health who have accepted the responsibility *1)* for providing technical advice on research grant applications to the National Advisory Councils and the Surgeon General, and *2)* in conjunction with the Councils, for surveying as scientific leaders, the status of research and training in their fields in order to determine areas in which additional activities should be initiated or expanded.

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INTRODUCTION

J. Murray Luck

From the Department of Chemistry, Stanford University, Stanford, California

It is my pleasure to introduce the panel discussion being held this evening, to tell you in a very few minutes something of its background, and to introduce to you the participants.

This is a working conference. It arises from the growing application in recent years of radioactive and stable isotopes to elucidate all sorts of problems in the experimental sciences. Some of the more challenging and, I may add, vexatious problems are emerging from the use of isotopically-labeled large molecules. With these we shall be concerned.

Much could be said in support of working conferences such as this in which the difficulties confronting those who are using labeled proteins may be aired, the problems may be considered critically, and the participants may learn from one another. We are indebted to the Division of Research Grants of the National Institutes of Health and to Dr. Gutman, the Chairman of the Metabolism and Nutrition Study Section, for making this conference possible.

I might also add, as a further introductory note, that the Metabolism and Nutrition Study Section, and I presume other study sections within the Research Grants Program of the National Institutes of Health, has before it an increasing number of applications for research grants-in-aid for work in this broad field; I suspect that the wise, judicious and careful appraisal of these applications is proving to be of increasing difficulty. It is difficult because the problems themselves are constantly in need of more precise definition and clarification, and because the hazards that attend the interpretation of the experimental data are not always appreciated.

In calling a working conference of this sort, the Metabolism and Nutrition Study Section may indeed be eager to have these problems presented, and the difficulties critically aired, so that as applications for support of research in this field come before the Study Section, it may be possible to consider these with all the wisdom that they very properly deserve. We hope that the participants in this discussion will feel encouraged to speak very freely and critically about the way they regard this area of research, and that they will present the difficulties as they see them. I am sure that when we leave this room, all of us here will go away somewhat better informed and more appreciative of the complexities of research in this area than we are at the present moment. With equal certainty, I know that some very helpful ideas will emerge.

This leads me to say just a word about the arrangements for the presentation of the formal papers. None of the speakers has been assigned a sharply defined topic which the Chairman could present to you as an exact delineation of the subject to be considered. But perhaps in a more general way, I can tell you that Dr. Oncley, Professor of Biological Chemistry at the Harvard University Medical School, will be our first participant and will give us the chemical background; we shall be presented with some of the solid chemical considerations upon which, from a very fundamental point of view, the work in this field must rest—whether or not it rests there now is another matter.

Following Professor Oncley, there will be papers by Dr. Howard Armstrong, who is Professor of Medicine at the University of Illinois Medical School; by Dr. Solomon Berson, Chief of the Radioisotope Service of the Veterans Administration Hospital in the Bronx, New York, who has worked extensively in this field; and, as the concluding contribution, a paper by Dr. Robert E. Olson, Professor of Biochemistry and Nutrition in the Graduate School of Public Health of the University of Pittsburgh.

The whole program is intended to be informal, to be critical, and to permit us to go away from here with an appreciation of the nature of the problems, and perhaps with some new and brilliant ideas for their experimental solution.

When we talk about investigations in this field, we start from a recognition of the fact that the most straight-forward research has been on problems that necessitated the use of labeled substances of small molecular size. It involved pure compounds that could be rigorously characterized; the incorporation of these into other

simple molecules amenable to isolation, purification and analysis could be pursued with directness and without ambiguity; the reactions studied were comparatively simple; and so-called 'turnover' and 'uptake' could be discussed with assurance. From this solid ground we have now risen to those more nebulous and stratospheric regions in which radioactive isotopes and stable isotopes, too, of course, are used in research with macromolecules of uncertain structure, of dubious stability and of doubtful purity. We apply them in reactions studied *in vitro*, and we study them with impunity in the complex environment and metabolic mills of the whole organism. These macromolecules, the proteins for example, do not lend themselves to the same ease of isolation and purification as do small molecules. Perhaps it is this lack of cordial cooperation on the part of the macromolecule and its richness in uncertainties that are at the root of our difficulties.

And so it is to the complexities of the problems that are inherent in the use of these large molecules, the proteins, that this symposium will be devoted.

CHEMICAL CONSIDERATIONS[1]

JOHN L. ONCLEY

From the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

MY CONTRIBUTION to this symposium is to be a discussion of some of the problems that must be faced in using protein molecules for tracer studies, from the viewpoint of a protein chemist. These remarks will be centered upon two major points: *1*) the preparation of the protein under consideration and *2*) the iodination of this material.

The whole question of protein purity is one which we will not attempt to critically examine here. We must certainly admit that protein 'purity' is difficult to define, and almost impossible to attain. However, I do not think that this is one of the principal problems in most tracer studies, since members of a 'family' of closely related protein molecules will probably be metabolized in a more or less similar fashion, and minor amounts of impurities will in general not greatly influence our results. More important is the question of protein denaturation. Here too we are dealing with a complex problem where it is difficult to accurately define even the term 'denaturation'. Certainly a denatured protein is not necessarily one that no longer will go into solution. An insoluble product is, of course, a kind of end result of this process, and fortunately an easy result to recognize. On the other hand, there are many clear looking protein solutions which contain large amounts of protein whose properties are unlike those of the native material in important aspects. Serum albumin represents a protein where one can often (and perhaps always) find small amounts of a faster sedimenting component during ultracentrifugal analysis, and there is considerable evidence that such material is removed from the circulating plasma at a considerably accelerated rate as compared with the native protein. There may also be a considerable difference in the rates of removal of such protein components by the normal and the pathological subject.

Electrophoretic homogeneity of a protein gives little or no evidence of homogeneity in regard to denatured protein; nor does electrophoresis alone give sufficient evidence for a single protein component. There are at least four completely different protein components of plasma which have nearly identical mobilities at pH 8.4 characteristic of β_1-globulin. We certainly can recognize the β-lipoprotein (or perhaps even two or more β-lipoproteins), the β_1-metal combining protein, and two other β_1-globulins which contain little or no lipid and do not interact specifically with certain metal ions. Fortunately, they can easily be distinguished in the ultracentrifuge. On the other hand, protein preparations which appear homogeneous, or nearly so, in the ultracentrifuge, are often found to contain several components when examined by electrophoretic methods. Thus a protein must be homogeneous by both of these criteria to have a reasonable expectation of representing a homogeneous material. Indeed, we should also require even other tests of homogeneity—uniform solubility and constant chemical composition, for example—before we can be satisfied that it is a single component.

The reactions of protein with iodine are quite complicated, and have been studied by a number of workers. In view of several recent studies of this reaction, we will not attempt here to do more than mention a few of the more obvious aspects of this reaction. For more critical analysis, recent studies by Hughes and Straessle (1), McFarlane (2) and Hughes (3) should be consulted. The following would seem to be the more important conclusions of these and other studies:

Various iodination reagents are known to react with several kinds of groups in most proteins. Reaction with the phenolic groups (tyrosyl residues) would seem to be the best goal for the introduction of iodine into the molecule, although certain easily oxidized residues, especially sulfhydryl groups, are

[1] This work has been supported by funds of Harvard University, by grants from the National Institutes of Health, and by contributions from industry.

probably the first to react with small amounts of reagent. (Studies involving initial reaction with small amounts of other oxidizing reagents which might react with these most easily oxidized groups, followed by iodination reagents might avoid this complication, but to the best of my knowledge have not been carried out.) The reagent of choice would appear to be I_3^-, buffered so that the reaction medium will be in the pH range from 9.0 to 9.5. The concentration of iodination reagent should be as low as possible, and the protein concentration should be in the range from 2 to 10% whenever possible. The reactions should be carried out with care to avoid local excess of reagents. The temperature should be maintained at very nearly 0°C.

By using such conditions, Hughes and Straessle were able to prepare iodinated human serum albumin which could be crystalized in good yield, and McFarlane has shown that quite reproducible half-lives can be obtained when various specific methods are used, so long as he uses the principles outlined above.

Removal of uncombined iodine must also be carefully accomplished. Dialysis of the resulting protein solution under conditions of maximum stability for the protein is usually the method used, but careful use of ion-exchange resins which will replace iodide ions by chloride or acetate ions has been found to be satisfactory and less time-consuming for at least some proteins. Some studies should also be carried out to be certain that a strong binding of iodide ions by the protein has not led to the incomplete removal of such reagent during the above procedures. The use of trichloracetic acid, or other protein denaturing reagent which should not free iodine actually incorporated into tyrosyl residues, as an analytical test for the absence of iodide ion is recommended.

It is very important to study carefully the results of any particular iodination procedure in order to know that complicating reactions have been held to a minimum. The use of the ultracentrifuge as an analytical method which can show the absence of appreciable quantities of molecules of altered size or (to some extent) shape has been of considerable value. Careful studies of the reproducibility of the half-lives obtained when materials which have been iodinated under varying conditions and to different amounts are injected into experimental animals are also of much value. It should also be pointed out that a method quite suitable for the iodination of a quite stable protein, such as serum albumin, may not be satisfactory when applied to a protein which is less stable, or which may react with iodine in a different manner.

Another problem pertinent to the iodination of proteins is the amount of homogeneity in regard to iodination which we will obtain under different conditions. Ideally we would like to obtain material which uniformly contained only one or two iodine atoms per mole of protein, since this would result in the least possible alteration of the protein. The statistical distribution of label if we consider a protein with a given number of identical reactive sites can be easily calculated from equations derived from probability considerations. We shall assume that a second label and other subsequent labels have the same likelihood of reaction as the first, except with the group already occupied which we will consider unreactive to any further extent. These conditions are probably not true for the iodination of tyrosyl residues, since Hughes and Straessle have shown that a second iodine is more likely to react with a mono-iodotyrosyl residue than with an unmodified residue, but this assumption simplifies our calculation, and the actual case will probably be only slightly worse than we calculate here, especially if we consider iodination of only a few residues among many capable of reaction.

We will define the following quantities:

m = maximum number of reactable residues
n = residues actually reacted
R = groups introduced per mole of protein
C_n = concentration of derivative containing n groups
nC_n = radioactivity of derivative containing n groups
$\sum nC_n$ = total radioactivity, where n varies from 0 to m

We can then easily calculate the fraction of the radioactivity contributed by molecules containing n groups from the equation

$$nC_n / \sum nC_n = \frac{m!}{n!(m-n)!R} (R/m)^n (1 - R/m)^{m-n}$$

TABLE 1. STATISTICAL EFFECTS DURING PROTEIN IODINATION*

% of radioactivity in molecules containing n atoms of iodine

Iodine atoms/mole protein in final product, R	6/1	4/1	2/1	1/1	1/2	1/4	1/6	1/10	1/20	1/50
Residues reacted, n										
1	0	2	13	38	62	78	85	91	95	98
2	1	7	28	38	30	19	14	9	5	2
3	4	15	28	18	7	2	1			
4	9	21	18	6	1					
5	15	21	8	1						
6	18	16	3							
7	18	10	1							
8	15	5								
9	10	2								
10	6	1								
11	3									
12	1									

* Calculated from *equation 1* for a protein with m = 24 reactable groups.

TABLE 2. STATISTICAL EFFECTS DURING PROTEIN IODINATION*

% of radioactivity in molecules containing one atom of iodine (n = 1)

Maximum no. of reactable residues, m	Iodine atoms/mole protein in final product, R			
	1/1	1/10	1/20	1/50
1	100	100	100	100
2	50	95	98	99
4	42	93	96	99
8	39	92	96	98
12	38	91	96	98
24	38	91	95	98
100	37	91	95	98

* Calculated from *equation 1* for proteins with different reactable residues, m.

where m, n, and R are given various values. Table 1 is for the case where m is 24, and where R is varied from 6/1 to 1/50. In table 2 we have varied m from 1 to 100 and R from 1/1 to 1/50. These values cover the range of reaction of interest to us in the iodination of serum albumin. Although albumin contains 18 tyrosyl residues, each theoretically capable of reacting with two iodine atoms, it would appear that some of these residues are considerably less reactive. The number 24 is only a guess, but we will show that the total number of residues is not very important when we wish to react only a few groups.

Table 1 shows that very heterogeneous products will be obtained unless we can aim at very low levels of modification. If we attempt to add say 6 atoms of iodine per mole of albumin, we get appreciable amounts of activity in fractions containing up to 10 or so atoms. At an average level of 2 atoms of iodine, we find 13% of the total radioactivity in molecules containing 5 or more atoms of iodine, and at an average level of 1 atom we find 8% of the total radioactivity in molecules containing 4 or more atoms. Only when we have aimed at a modification of 1/10 do we have less than 10% of radioactivity in molecules containing more than 1 atom of iodine. It can be seen from table 2 that these calculations are relatively insensitive to our choice of the maximum number of reactable residues, m, so long as we are using small ratios of iodine per mole of protein.

If we attempt to use very small ratios of iodine per mole of protein, it is very likely that most of the added reagent will be used up in deleterious side reactions (with a few very reactive groups like sulfhydryl) and not result in an introduction of the iodine into the protein molecule. If some other mild oxidizing agent could be first used to react with these extraneous groups of a protein, as was suggested earlier, then it might be possible to use ratios of modifying reagent in the range of 1/10–1/50 with a great gain in homogeneity. (At the present time this procedure would not be practical.)

Another type of study of protein half-lives involves the preparation of a heterogeneous mixture of marked proteins, perhaps by the feeding of marked amino acids or other precursors, from which we must isolate the particular purified protein under study. Here our criteria of protein purity are equally important, but can be applied at the end of the experiment. Here we are not so concerned by protein denaturation if we can be sure that a specific protein entity is recovered, and this may be a method of choice for a protein of very labile character. The use of specific antisera can be very helpful here, but again we must prove the specificity of the antisera to a given homogeneous protein. Thus in the above procedures we are not able to avoid the necessity for careful studies of homogeneity.

We have not attempted to define the amount of iodine which can be introduced into a protein without a significant change in its metabolism or function, or with the problems of the reutiliza-

tion of protein fragments, since these will be treated by other members of this symposium. We have concentrated our attention on a few of the problems of obtaining and maintaining homogeneous preparations of proteins for studies of this type. The problem is not an easy one, and we would like to leave this discussion with an admonition to take very little for granted in this work, and to carefully study the procedures used for each individual protein investigated.

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2. McFarlane, A. S. *Biochem. J.* 62: 135, 1956.
3. Hughes, W. L., Jr. *Ann. New York Acad. Sc.* In press.

FAILURE OF SUPPLEMENTARY DIETARY AMINO ACIDS TO MINIMIZE THE EFFECT OF S^{35} REUTILIZATION ON THE DIE-AWAY PLOTS OF AUTOSYNTHESIZED ALBUMINS AND GAMMA GLOBULINS[1]

S. Howard Armstrong, Jr., David Bronsky and Samuel Hyman

From the Hektoen Institute of Medical Research, Cook County Hospital, and the Departments of Medicine, University of Illinois College of Medicine and Northwestern University Medical School, Chicago, Illinois

In the interpretation of rates of turnover of plasma protein fractions in disease states, it would be a great advantage to be able to measure rates for proteins synthesized by the diseased subject himself.

Thus, while the rheumatoid arthritic and the normal subject differ only slightly in 'apparent turnover times'[2] for normal pooled gamma globulins *in vitro* labeled with I^{131} and intravenously injected, this fact carries no guarantee that the rheumatoid is not turning over a fraction of his own abnormal gamma globulins at quite a different rate than the normal subject (1).

Our group's three years of work (2–5) in seeking valid apparent turnover times of proteins autosynthesized following the oral S^{35} label carry for us more discouraging implications than are suggested by the careful studies of Volwiler and his colleagues (6). Our consistent finding of longer apparent turnover times of the autosynthesized protein fractions than those of comparable intravenously injected fractions (whether *in vitro* I^{131} iodinated or *in vivo* S^{35} labeled) emphasize the point made by Luck (7) in his recent editorial: "One can be reasonably sure that recycling will constitute a serious problem in experiments in which albumin-S^{35}, C^{14} or N^{15} is employed."

This probability of continuous reutilization has led McFarlane and his colleagues in England to his recently published animal comparisons of disappearance rates and pool sizes of proteins both labeled *in vitro* with I^{131} and autosynthesized by another animal after C^{14} ingestion. These workers comment "This method . . . does not entirely eliminate recycling of the label but reduces it to negligible proportions" (8–10).

Luck's editorial, however, states "It is common practice to minimize this source of error by the administration of relatively large quantities of unlabeled cystine, methionine, or such other amino acids which, by entry into the amino acid pool, will dilute out the labelled amino acid released from the protein during metabolism. How effective this device is cannot be determined."

The results presented here relate to: *1*) an appraisal of the suggestion of Volwiler's group that, on intermediary metabolic grounds, cystine will be less subject to recycling than methionine; *2*) an appraisal of the device of dietary unlabeled amino acid supplements; *3*) whether the intravenous route is preferable to the oral route for administration of the labeled amino acids.

RESULTS

Die-Away Plots Following Oral S^{35} Methionine Without Unlabeled S Amino Acid Supplementation. Customarily the slope of the first 5 days of a die-away plot is considered chiefly representative of equilibration of the intravascular labeled plasma protein with the extravascular pool. The slope for the next 10 days or so is considered chiefly characteristic of the rate of metabolic degradation of the labeled protein.

In our previously published studies we have reported a very frequent flattening of the slope of the die-away plots which may begin late in this 'metabolic period' and, particularly following oral S^{35} labeled yeast, may become quite striking between the 20th and 40th days. More rarely have we noticed such flattening with an intravenously injected *in vitro* labeled protein.

In figure 1 are presented albumins and gamma

[1] These studies have been supported by Grant H-1511(C,C2) from the National Heart Institute, U.S. Public Health Service. Publication in extenso of these results is in preparation for the series in the *J. Lab. & Clin. Med.*

[2] In this report 'apparent turnover time' is used in preference to 'biologic half life'. This is equal to the biologic half-life divided by the natural logarithm of 2.

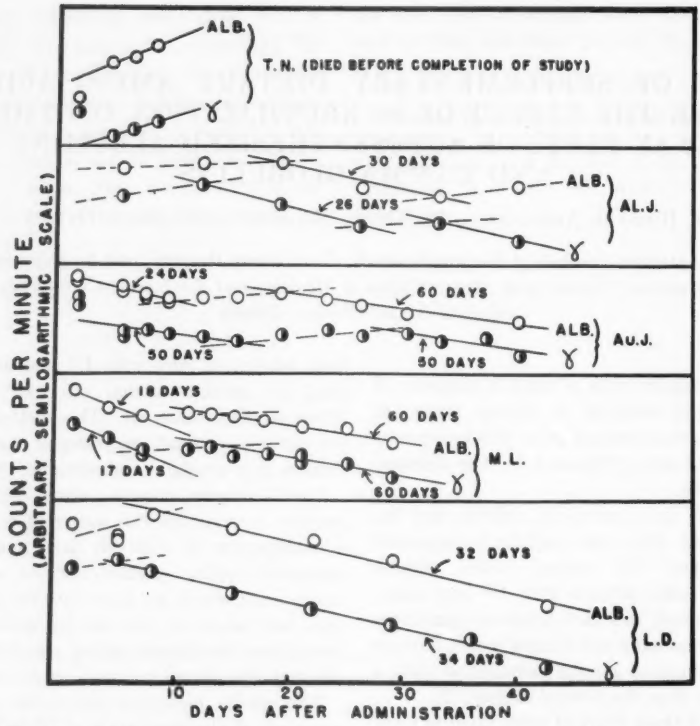

FIG. 1. Albumin and gamma globulin die-away plots in 5 patients with abnormal gastrointestinal tracts following methionine S^{35} ingestion.

T. N. (top): chronic pancreatitis; serum albumin 0.7 gm%. *Al. J.* (second from top): postoperative total pancreatectomy; serum albumin 2.8 gm%. *Au. J.* (third from top): chronic calcific relapsing pancreatitis; serum albumin 3.4 gm.%. *M. L.* (fourth from top): chronic calcific relapsing pancreatitis; serum albumin 3.8 gm%. *L. D.* (bottom): sprue; serum albumin 2.4 gm%.
Apparent turnover times corresponding to slopes of selected segments are indicated next to the arrows.

globulins die-away plots of five subjects. The subjects represent a variety of nutritional problems with albumin levels ranging from 0.7 to 3.8 gm%. Some clinical details on the subjects are given on the legend to the figure; these details are less important to use here than the form of the die-away plots, particularly in the first five days representative of 'equilibration' when the slope should, in the absence of isotope reutilization, be sharply downward. The top two and bottom subjects have *rising* specific activity levels for albumins and gamma globulins during these first days. The two middle patients in the figure have specific activity levels which, although showing an initial decline in the mixing and metabolic periods, show some evidence of a rising level before a final falling slope. Whereas the top two patients could not be described as clinically in the 'steady state', the bottom three could so be described. Therefore, these roller-coaster die-away plots clearly preclude any graphical translation of slopes into apparent turnover times. The initially encountered rising slopes suggest a label recycling so great as to obliterate completely the downward slope ordinarily characteristic of the equilibration period. We thus concur in the suggestion of Volwiler's group that the oral S^{35} methionine label is not adaptable to clinical studies of plasma protein fraction turnover by die-away plot.

Comparison in a Hypoalbuminemic Subject of the S^{35} Methionine Oral Label with the I^{131} Intravenous Label for Albumins and Gamma Globulins. In figure 2 are presented three sequential studies on a patient whose nutritional hypoalbuminemia was due to a faulty intestinal surgical anastomosis. It will be noted in both studies following oral S^{35} methionine *a*) the albumin die-away plots show either unchanging or *increasing* specific activity for almost three

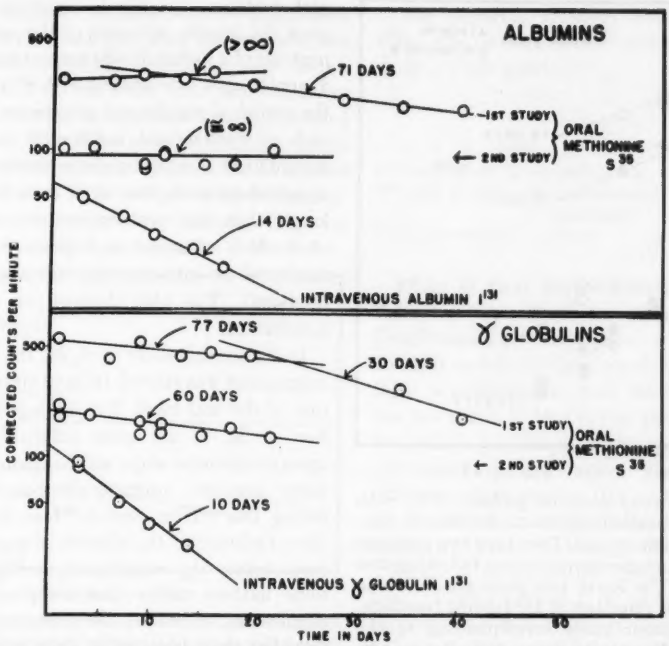

FIG. 2. Comparison of die-away plots of albumins and gamma globulins following oral S^{35} methionine and following intravenous I^{131} labeled protein fractions (Patient *W. S.*). The top two lines in the upper and lower diagrams represent duplicate studies with the oral isotope and show in the first study terminal dropping off rather than a terminal flattening of the die-away plot. A glance shows the great difference between the I^{131} and S^{35} slopes in this subject. Corresponding apparent turnover times are indicated as in fig. 1.

weeks, and *b*) the comparable die-away plots for the gamma globulins while not rising or flat show a very slow decrease with time. By contrast, slopes of the plots following intravenous I^{131} labeled proteins show steep declines. For albumins this corresponds to an apparent turnover time three or four days shorter than is usually found in normal subjects. For gamma globulins the apparent turnover time is shorter than in the normal, but less so.

This type of subject illustrates the most accentuated discrepancies between the I^{131} and S^{35} labels that we have encountered and further confirms our dissatisfaction with oral methionine as a valid label for clinical investigation.

Comparison in a Hypoalbuminemic Subject of the S^{35} Cystine Oral Label with the I^{131} Intravenous Label for Albumins and Gamma Globulins. In figure 3 are illustrated for another nutritionally hypoalbuminemic patient a comparison between I^{131} and S^{35} apparent turnover times where the oral label has been L-cystine. Here the slopes during the metabolic period yield apparent turnover times which are significantly shorter than are usually found after the oral S^{35} isotope but still considerably longer than with comparable findings with the I^{131} intravenous label. While this result is consistent with the Volwiler group's suggestion that cystine may be preferable to methionine from a recycling standpoint, clearly cystine lessens but does not eliminate the discrepancies between apparent turnover times obtained by the S^{35} and I^{131} methods in the same subjects.

Comparison of Effect of Dietary Unlabeled S Amino Acid Supplements on Apparent Turnover Time of Plasma Protein Fractions Immediately and Months After Oral Administration of the S^{35} Label. We have previously reported the failure of dietary unlabeled oral methionine and cystine supplements to influence the very flat albumin and gamma globulin die-away plots of one uremic subject (2). While consistent with the report of Madden and Gould (11) on fibrinogen, this finding provides no evidence that such supplementation effectively minimizes isotope reutilization.

The following results illustrate our search for

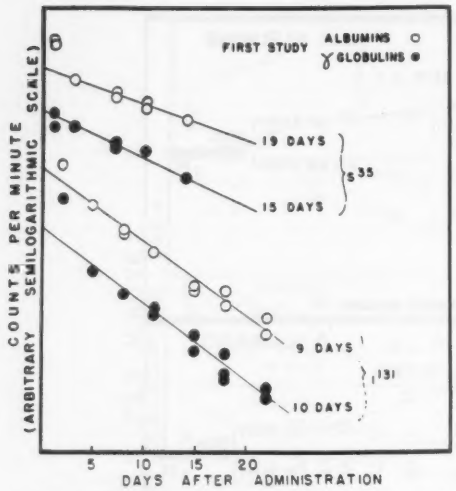

FIG. 3. Die-away plots on patient *T. C.* with nutritional hypoalbuminemia secondary to prolonged low protein intake. The upper two plots are for albumins and gamma globulins following oral S^{35} L-cystine. The lower two plots are following the intravenous injection of I^{131} labeled fractions. Apparent turnover times corresponding to the slopes during 'metabolic decay period' are indicated to the right of the plots.

such evidence. The top die-away plot of figure 4 gives the specific activities of the unfractionated proteins of a metabolically normal subject, *J. M.*, 8 months after oral labeling with S^{35} yeast. During the period of supplement administration (2.5 gm each of cystine and methionine per day) the immediately steepening slope corresponds to the apparent turnover time of 17 days (about 4 days longer than the mean apparent turnover times of *J. M.'s* albumins and gamma globulins as measured by intravenously injected I^{131} labeled fractions). The plot flattens on cessation of supplement.

In the second study on *J. M.* the same dosage supplement was started 18 days after administration of the oral label. The findings (bottom section of fig. 4) are quite different. The initial upward albumins slope and flat gamma globulins slope suggests intensive isotope reutilization during this mixing period. More important are these findings: *a*) the albumin slope turns downward before the supplement is started; *b*) the slope flattens rather than steepens during the supplement; *c*) neither the albumins nor gamma globulins slope becomes as steep as the slope for unfractionated proteins when the supplement is given 8 months after the oral label.

Following the analogy of Schoenheimer and

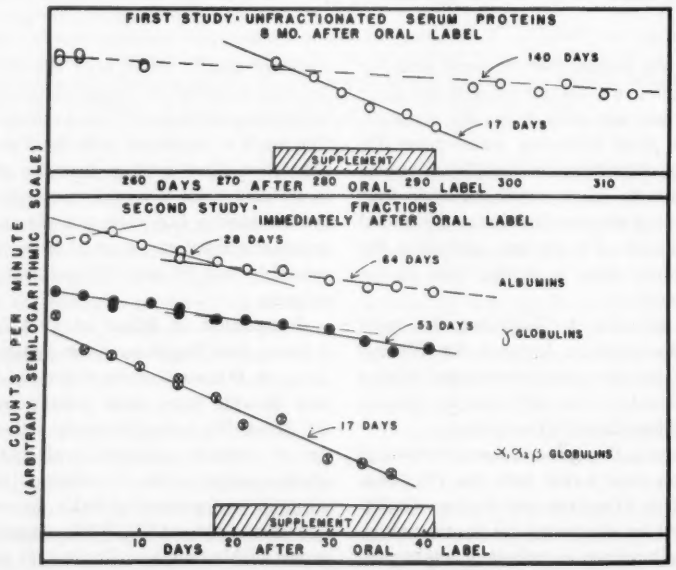

FIG. 4. Comparison of effect on die-away plots of oral **unlabeled S amino acid supplementation** started 8 months (*top section*) and 18 days (*bottom section*) after oral dose of S^{35} amino acid. (Patient *J. M.*). Because of low specific radioactivities, fractionation in the first study (*upper*) was impractical. Fractions prepared as described elsewhere are plotted for the second (*lower*) study.

his group's classic studies on N^{15} (12), these findings suggest that only after the oral label has become well distributed through the body's tissue protein stores with very slow turnover rates can recycling be repressed by oral unlabeled supplements. This takes months, not days. When after 2 weeks or so such organs as the liver have specific activities about as high as those of simultaneously obtained plasma proteins (4), oral supplements at this dosage seem ineffective.

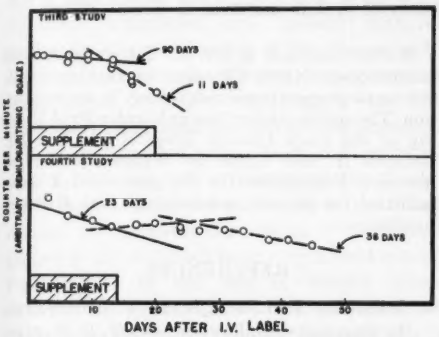

FIG. 5. Two sequential studies in same subject (*J. M.*) to illustrate immediate oral supplementation (5 gm cystine, 5 gm methionine/day) on albumin die-away plots following intravenous S^{35} cystine. Apparent turnover times are indicated as in previous figures.

The following apparent turnover times illustrate the interpretative dilemma for subject *J. M.* at this point.

Label	Days Albumins	Days Gamma globulins
S^{35} oral yeast (no supplement)	64	40
S^{35} oral cystine (no supplement)	28	41
(supplement)	64	53
I^{131} intravenous fraction	17	12

Effect of Oral Supplementation Immediately Following Intravenous Injection of S^{35} Cystine. Confirmation of the figure 4 experiment in another metabolically normal subject still left these possibilities: *a*) that the oral supplement was too small; *b*) that it was given too late after the initial oral label; *c*) that the intravenous route might be preferable to the oral route for labeling administration (in order to minimize the length of time significant label concentrations were circulating through the liver.)

Figure 5 presents two further albumins die-away plots on subject *J. M.* of figure 4. The oral supplement was increased to 5 gm of cystine and 5 gm of methionine a day. This was started immediately following the intravenous injection of the S^{35} labeled cystine. It will be noted: *a*) in neither study was a single characteristic die-

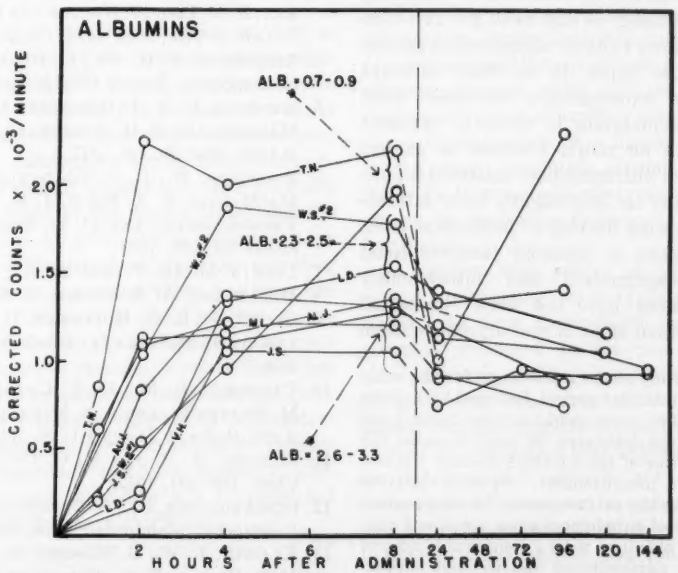

FIG. 6. Albumin S^{35} specific activities (uptake plots) following oral S^{35} methionine (500 μc) without unlabeled amino acid supplementation in 9 subjects with albumin levels ranging between 0.7 and 3.3 gm%. (Specific activities are corrected to a single arbitrary standard of counts/min/gm albumin as of day of isotope administration.)

away plot slope seen;[3] *b*) the two die-away plots are far from superimposable, although no clinical change was noted between experiments. (These findings have been duplicated in another metabolically normal subject.)

Specific Activity Uptake Plots. Our studies (fig. 6) confirm Kinsell and his coworkers' (13) observation on a case of idiopathic hypoproteinemia, namely, that the initial maximum specific activity level following a standard oral isotope dose can prove much higher in the presence of hypoalbuminemia than with normal serum albumin concentrations. Because of the possibility that this is related to the diminished apparent exchangeable albumin pool (demonstrated by the I^{131} method in the hypoalbuminemic subjects), we do not feel at the moment that uptake studies can supplant our unsatisfactory die-away plots as a crude clinical measurement of rates of synthesis of a specific protein component. We as yet have had no experience with methods comparable to the constant exposure technique used in the nucleic acid studies of Swick *et al.* (14).

SUMMARY AND CONCLUSIONS

The use of S^{35} cystine, whether given orally or intravenously, whether or not accompanied by oral supplements of unlabeled S containing amino acids in dosage as high as 10 gm/day, have provided die-away plots for albumins and gamma globulins whose slopes do not have sufficient consistency or reproducibility to permit valid metabolic interpretation in terms of apparent turnover times for serum albumins or gamma globulins. Even with immediate unlabeled supplement the results are only slightly more satisfactory than following the oral S^{35} methionine label. The discrepancies in apparent turnover times between autosynthesized and intravenously injected fractions have not been eliminated. While an apparent effect of oral supplementation has been noted on the slope of die-away plots some months after the oral S^{35} amino acid label, a method based on this finding would be hardly of value for clinical investigation. As a rule, one wants turnover data in the immediate future and not after months in which unpredictable clinical phenomena can occur. In our opinion these findings make it very unlikely that oral isotope administration, except in the most crude empirical fashion, is going to prove a valid tool for the study of protein turnover in disease states.

We are indebted to Dr. D. Tabern of Abbott Laboratories, North Chicago, for making available to us preparations under study in his institution. The senior author thanks Warden Fred Hertwig of the Cook County Hospital and the Dr. Leonard H. and Louis D. Weissman Medical Research Foundation for the provision of basic facilities for protein research at the Hektoen Institute.

[3] The top study shows a flat slope for the early part of the supplement period, followed by a sharp steepening. This study could not be carried out further than the indicated 20 days because the specific activities of the proteins became too low for significant measurement. (Specific activity levels following the intravenously labeled protein with this type of supplementation are about one-third of those obtained with a comparable dose of isotope orally administered and without supplement.) In the bottom study, while a slope from the 5th to the 12th day gives an apparent turnover time of 23 days, this value is still about 6 days longer than a comparable figure with this subject as measured with I^{131} albumin.

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DISTRIBUTION AND METABOLISM OF I^{131} LABELED PROTEINS IN MAN

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DR. ARMSTRONG has illustrated to us the complications introduced into protein turnover studies by the reutilization of S^{35}. Similar difficulties are likely to be inherent, to a greater or lesser degree, in studies employing N^{15} and C^{14}. These particular disadvantages are not shared by I^{131} as a protein label, since if thyroidal accumulation of I^{131} is blocked by administration of relatively large amounts of stable iodide, the iodine released by metabolic degradation of the labeled protein is not reutilized to any significant extent. Furthermore, in the case of labeled serum proteins, the iodine released is almost quantitatively excreted in the urine at a rate which is quite rapid compared to the rate of protein degradation. Therefore the latter becomes rate limiting for the appearance of protein-free I^{131} in the urine so that the urinary excretion of I^{131} can serve as a measure of protein turnover in such cases.

I^{131} labeled serum albumin may be used to demonstrate some of the methods employed in the application of I^{131} labeled proteins to turnover studies. Of interest is its rate of transfer between anatomic or physiologic compartments, its ultimate distribution among those compartments, its rates of degradation and synthesis (which are equal in the steady state) and the anatomic sites at which these processes take place. Some of the more basic questions regarding the mechanisms governing these processes are not readily forthcoming from studies in the whole animal and require less complex and more readily controllable systems. Nevertheless a fair amount of information has been obtained from observations following intravenous administration of I^{131} labeled proteins in the intact animal. Since our experience has been primarily with man, the present discussion will be restricted chiefly to experiments in this species.

Following intravenous administration of I^{131} labeled albumin, the plasma concentration (plotted logarithmically as a function of time) falls in a curvilinear fashion at first and then approaches a straight line (1). The very earliest changes in concentration are related to mechanical mixing in the plasma which is not complete for some 5–10 minutes at least (2). Doctors Bauman and Rothschild (3) in our laboratory have demonstrated directly that in those regions of the body where blood flow is relatively small compared to blood content, particularly in the lower extremities, uniform mixing frequently takes 7–8 minutes, while in those organs with high rates of blood flow, mixing is complete within a minute or two.

After the mixing period the plasma concentration continues to fall, initially at a rate of about 4% per hour, with a gradually decreasing slope until, after 4–7 days, a straight line decrease is observed (4). During the period of changing slope, the fall in plasma concentration reflects equilibration of albumin-I^{131} between intra- and extravascular albumin pools, as well as the metabolic degradation of albumin. The final straight line decrease represents the rate of degradation. Since the initial rate of loss from plasma is about 4% per hour and total circulating albumin averages about 135 gm, we conclude that some 5 gm of albumin or more passes across the capillaries each hour or that an amount equal to the entire circulating albumin passes daily (table 1). It is probable that return to the blood stream is almost exclusively by way of the lymphatics.

In subjects with ascites or other large and slowly equilibrating pools of extravascular albumin, the time required for equilibration is generally prolonged. In one case of decompensated cirrhosis with ascites where the tagged albumin was administered intraperitoneally, mixing was not complete even after 3 weeks (5). It must be emphasized that unless sufficient time for distribution is allowed, not only will the exchangable albumin pool be underestimated but also a greater error (overestimate) will be made in the rate of metabolism, since the fall in plasma concentration will be contributed to by continued distribution as well as by degradation.

In calculation of the total exchangeable pool of serum albumin, it has been a common practice,

TABLE 1. APPROXIMATE AVERAGE VALUES OBTAINED WITH ALBUMIN-I^{131} FOR DISTRIBUTION AND METABOLISM OF ALBUMIN IN NORMAL HUMAN SUBJECTS

Rate of transcapillary passage	5 gm/hr
Total exchangeable albumin	5 gm/kg
Intravascular	40%
Extravascular	60%
Skin	18%
Muscle	15
Heart, lung, liver, kidney, spleen	4
Unaccounted for	23
Half-time for turnover	18 days
Fractional rate of degradation	4%/day
Synthesis	0.2 gm/kg/day

in studies of this type, to extrapolate the metabolic straight line portion of the curve to zero time (fig. 1) and to consider this to represent the concentration which would exist if the tracer were instantaneously distributed throughout its final volume of distribution. But this would be correct only if the rate of degradation were constant throughout the distribution period which would mean that the rate of degradation was the same in all compartments. Since this is not the case, an avoidable error is introduced by this practice. To clarify this point let us suppose that degradation takes place only in the most slowly equilibrating extravascular pools. Then, clearly, virtually no degradation would have occurred during the very early period since the tracer had not yet entered these pools in significant quantities. The extrapolation should therefore have been flattened out as it approached zero time. Conversely, if degradation occurred primarily in the plasma itself or in rapidly equilibrating compartments, the rate of degradation would be greater during the early period and the extrapolation should have bent upwards as it approached the ordinate. It has been shown that the rate of degradation parallels the concentration in the rapidly equilibrating compartments (5) so that the latter situation is the one which appears to obtain in the body. These errors can be minimized by evaluating the space of distribution from the usual 'indicator dilution' formula,

$$\text{volume of distribution} = \frac{\text{amount injected} - \text{amount excreted}}{\text{plasma concentration}}.$$

Perhaps of greater importance is that the calculation of total exchangeable albumin by this method also obviates errors arising from the presence of nonprotein bound radioactivity or rapidly degraded denatured components.

When a graph of the volume of distribution or its reciprocal, plotted as a function of time (for convenience termed a 'distribution curve'), reaches an horizontal asymptote, it indicates that no further increase in the space of distribution is occurring and that any further fall in the plasma concentration is related solely to metabolism. In the absence of nonprotein bound radioactivity or denatured material this method yields a value for total exchangeable albumin about 5% smaller than is obtained from the zero time extrapolation of the plasma curve. Since it can be shown from the theory of open compartment systems that the specific activities are not identical in all compartments at distribution equilibrium[1], even this method gives values slightly too high for total exchangeable albumin. However the error is probably no greater than 3% or so under most circumstances (APPENDIX). On the other hand, when loss from the plasma is extremely rapid, as in marked proteinuria, the estimate of total exchangeable albumin may involve a significant error. Gitlin, Janeway and Farr (6) have recently studied nephrotic children with marked proteinuria and have stated that specific activities in edema fluid may be 30 times as high as in plasma. These values are much too high to be resolved on the basis of mathematical theory alone (APPENDIX) and cannot be accepted without challenge. Nevertheless, they do serve to point up the difficulties in cases where loss of tracer from the plasma is rapid compared to the rate of return from extravascular regions. A similar problem has been encountered in the measurement of extracellular space with inulin and sucrose (7).

If the 'distribution curve' is resolved into its exponential components, it is almost always observed to have two such components in addition to the constant asymptotic value and can be described by the equation $A_e^{-\lambda_1 t} + B_e^{-\lambda_2 t} + C$. While the presence of two such components is evidence for the existence of at least two main groups of extravascular compartments equilibrating with plasma at distinguishably different rates, it must be emphasized strongly that the rates of these two exponential components are not the rates of equilibration of the two compartments

[1] Distribution equilibrium in these cases is defined as the state in which the ratios of specific activities among the various compartments remains constant.

with the plasma nor are the coefficients of these components a direct measure of the relative sizes of these compartments. However, since the sum of A + B does represent extravascular albumin, (A + B)/C is the ratio of extravascular to intravascular albumin, which in normal subjects averages 60:40 (4). This ratio is increased significantly in myxedema (8, 9) and in patients with massive ascites (4).

It was of interest to try to evaluate the anatomic distribution of the extravascular compartments. Earlier we had obtained evidence which indicated that the liver did not represent an important extravascular pool of albumin (10) and these observations were confirmed by examination at autopsy of the tissues of 2 patients who had received I^{131} labeled albumin 1 and 22 days respectively prior to death (11). It was found that the total extravascular albumin content of liver, kidneys, spleen, heart and lungs did not amount to more than 4% of the total exchangeable albumin of the body (11). From a comparison of the tissue/plasma ratios in the two subjects, it also appeared as if the liver, spleen and intestine had reached their equilibrium concentrations within 1 day but that the I^{131} albumin content of muscle, lung and several other tissues continued to increase significantly after this time.

These studies were extended by assay of muscle and skin biopsies from subjects who had received intravenous albumin-I^{131} at various intervals prior to surgical procedures. It was observed that not only was the final equilibration slow in these tissues but also that their albumin content accounted for more than half of the total extravascular pool. The relatively high albumin content of skin (amounting to about 25–30% that in plasma) might have been anticipated from its high extracellular fluid content. Altogether more than three fourths of the total body albumin could be accounted for in the plasma, skin, muscle and the major viscera indicated above (table 1).

Let us now turn from the distribution of I^{131} labeled serum albumin to its metabolism. In the steady state, once distribution equilibrium is attained, the fractional rate of fall in the plasma concentration should be equal to the fractional rate of loss from the body as a whole. Since the latter is reflected in the rate of urinary excretion, the rate of albumin degradation can be obtained from two independent sources of data, namely plasma and urine (4). However, changes in plasma concentration may be produced by factors irrelevant to the rate of protein metabolism, such as hemodilution or hemoconcentration

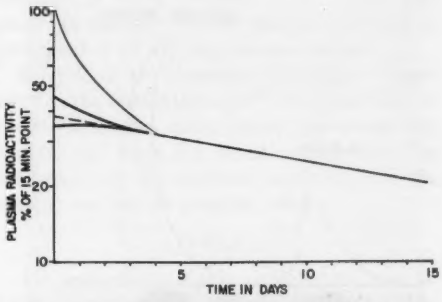

FIG. 1. Typical curve of plasma concentration of albumin-I^{131} as a function of time following intravenous administration. The broken line represents the extrapolation of the 'metabolic' segment of the curve. For the significance of the heavy curved lines above and below see text.

or shifts between extravascular and intravascular compartments. Under these conditions, interpretation of the plasma curve alone is very uncertain and employment of C^{14}, N^{15} or S^{35} is likely to lead to difficulty. Here, however, I^{131} labeled albumin remains useful. Since the renal excretion of nonprotein-bound I^{131} reflects the metabolic degradation of albumin, the rate of protein degradation can still be determined even in the absence of the steady state. Let us suppose, for example, that s subject excretes 300×10^{-3} μc I^{131} (nonprotein-bound) during a day when his mean plasma concentration is 1×10^{-3} μc I^{131}/ml. This indicates that he has metabolized the albumin content of 300 ml. of plasma. If the plasma albumin concentration is 4 gm/100 ml, the rate of metabolism is 12 gm/day. We might then speak of the 300 ml plasma/day as the 'metabolic clearance' of albumin so that the quantity of albumin degraded is equal to the product of metabolic clearance and the albumin concentration. This approach, originally used for evaluating the metabolism of endogenously synthesized thyroid hormone (12), was subsequently applied to the study of albumin metabolism in normal subjects rendered hyperthyroid by the administration of desiccated thyroid, 12–15 grains/day (9). Following institution of thyroid treatment there ensued a progressive increase in the rate of albumin degradation. However, because of an increase in plasma volume, a decrease in serum albumin concentration, and a shift from extravascular to intravascular compartments, the slope of the plasma curve itself was of limited usefulness in evaluating these changes. The 'metabolic clearance' technique made it possible to calculate the amount of

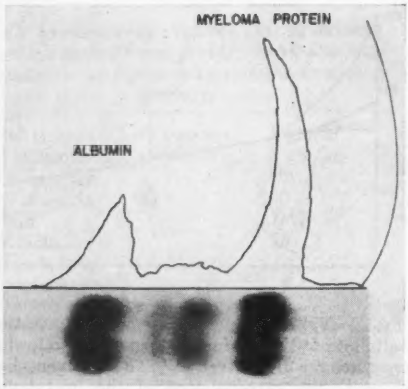

FIG. 2. Paper electrophoretogram of radioiodinated plasma of subject with multiple myeloma.

albumin degraded each day throughout the study. The total amount degraded during the period of thyroid treatment was obtained from the sum of the daily values. A second tracer dose of albumin-I^{131} was then injected for revaluation of the total exchangeable albumin which was observed to have decreased somewhat in all cases. From the values for the total amount degraded and the change in total exchangeable albumin, it was a simple task to calculate the amount synthesized during this period. Thus both the albumin degraded and the albumin synthesized were determined during a nonsteady state period.

The method employing the 'metabolic clearance' concept has further advantages. Not only is the determination of total exchangeable albumin unnecessary but since the degrading compartments are in rapid exchange with the plasma, complete distribution equilibrium is also not essential in this method. Values for albumin turnover obtained by all three methods—*1*) from plasma data alone; *2*) from urine data alone; *3*) from metabolic clearance—are generally in excellent agreement (9). In adult male subjects without abnormalities of protein metabolism, these methods indicate an albumin turnover of about 15 gm/day (4, 9, 13) which amounts to an average of 205–215 mg/kg/day (13).

The discussion thus far has related to I^{131} labeled serum albumin. Similar studies, of course, are feasible with any other protein which can be obtained in a reasonably homogeneous form, such as hormones, antigens etc. However, it is not necessary to employ a 'pure' protein to accomplish the same ends provided that the protein of interest can be identified in the iodinated mixture administered and in the body fluids after administration. For example, in a study of the metabolism of myeloma protein (14), the problem of separation and purification of the specific protein was avoided by iodination of the whole serum of a subject with a high concentration of myeloma (γ) globulin. The I^{131} labeled serum was then administered back to the original patient as well as to another subject with multiple myeloma who did not manifest any particular abnormality in the serum proteins but who did excrete a rather large amount of Bence-Jones protein in his urine. Plasma samples taken at intervals from both subjects were subjected to electrophoresis on paper strips which were then scanned for radioactivity (fig. 2). Concentrations corresponding to albumin and myeloma protein were determined by measurement of the areas under the respective peaks to yield data regarding the exchangeable pools and turnover rates of these proteins in donor and recipient subjects. Examination of protein-bound radioactivity in the urine did not support the concept of a precursor-product relationship between serum myeloma protein and Bence-Jones protein respectively.

In the few minutes remaining, I would like to call your attention to some of the pitfalls associated with the use of I^{131} labeled proteins. Early in our studies we were plagued by the presence of heterogeneities with respect to the rate of degradation of iodoalbumin. With some preparations, a rather large fraction was degraded rapidly, occasionally as much as 18–20% of the dose appearing in the urine as nonprotein-bound radioactivity during the first day (4). The rate of metabolism of the iodoalbumin subsequently showed a more or less gradual decrease to a rate of 4–5% per day after several weeks. These findings suggested that a fraction of the labeled albumin was damaged in preparation. With the development of better preparations we subsequently obtained fairly uniform rates of degradation at about 4%/day or less ($T\frac{1}{2} \cong 18$ days). These are sufficiently close to the rates obtained by London with N^{15} labeled glycine (15) and by Volwiler in his transfusion experiments employing biosynthetically labeled albumin S^{35} (16) to indicate that the I^{131} labeled proteins can give valid turnover data, especially when one considers that the slower rates obtained with the N^{15} and S^{35} labeled proteins are probably due to reutilization of those isotopes. More recently McFarlane

and associates (17, 18) have observed essentially the same rate of metabolism with carefully prepared albumin-I^{131} as with albumin labeled endogenously with C^{14} *in vivo* in the rabbit. However, reports have continued to appear wherein I^{131} labeled serum albumin is metabolized at rates of 5½–6% or more per day in normal human subjects (19). Although damage to the protein can occur during the process of labeling as the result of any number of chemical or physical effects depending on the method of iodination employed, our experience with alterations of I^{131} labeled insulin by self-irradiation (20, 21) stimulated us to investigate the effects of irradiation on the rate of serum albumin metabolism (22, 23). Lots of albumin were iodinated with small amounts of I^{131} and aliquots were then irradiated with a dose of 45 kilorad from a 1 Mev peak energy x-ray machine. This is about equal to the radiation dose absorbed in one week by solutions containing 700 μc I^{131}/ml. Irradiated and control preparations were handled in an identical manner except for the irradiation, and samples of each were administered to human subjects without known abnormalities of protein metabolism. When the irradiated solutions contained 0.2 mg protein/ml the damage was considerable as was manifested in an abnormally rapid rate of metabolism. Increase in the concentration to 20 mg/ml appeared to protect against the irradiation damage quite completely but at 5 mg/ml there was still some alteration of the protein which led to its more rapid degradation. Similar changes were observed when the source of irradiation was I^{121} in solution rather than x-rays. The protective effect of high concentrations can and should be utilized whenever possible and there is no reason not to do so in metabolic studies employing I^{121} labeled serum proteins. In certain instances, as in the use of labeled hormones, in order to be able to administer sufficient radioactivity for convenient assay and yet to remain within physiologic if not tracer levels of the hormone itself, it is necessary to employ preparations of high specific activity. Special precautions against radiation damage are then required since the alterations produced may be particularly troublesome. In the case of hormones such as insulin and glucagon, irradiation produces labeled products which bind to the serum proteins and long outlast the rapidly metabolized tagged hormones in the blood stream. Therefore it has been our practice, in cases where high specific activity preparations are essential, to add large amounts of serum albumin to the tagged hormone. In this way further irradiation damage is prevented or at least kept to a minimum.

In spite of the numerous difficulties encountered in the application of I^{131} labeled proteins to metabolic studies much useful information can be obtained when due attention is paid to the limitations of the methods employed and the various sources of possible error.

APPENDIX

The total exchangeable albumin pool may be treated as an open two-compartment system composed of *1)* plasma and rapidly exchangeable extravaseular spaces and *2)* slowly equilibrating extravascular spaces including edema fluid. Loss from the system as the result of metabolic degradation or proteinuria occurs from the first of these compartments. Although the assumption of two compartments represents a gross oversimplification, it would appear to be valid for the purpose of the present discussion.

If the total radioactive albumin in each of the compartments is A'_1 and A'_2 respectively and the total endogenous albumin is A_1 and A_2 respectively then at any time, t, after administration of radioactive albumin,

$$dA'_2/dt = -\lambda_{21} A'_2 + \lambda_{12} A'_1 \qquad (1)$$

and in the steady state

$$\lambda_{21} A_2 = \lambda_{12} A_1 \qquad (2)$$

where λ_{12} and λ_{21} are the rate constants for albumin transfer in the directions indicated by the subscript designations.
From *(1)*

$$\frac{dA'_2/dt}{A'_2} = -\lambda_{21} + \lambda_{12} \frac{A'_1}{A'_2} \qquad (3)$$

After distribution equilibrium (see footnote[1]) is attained, the slopes of disappearance in all compartments are identical and equal to K, the fractional rate of loss of total body radioactive albumin. Therefore, at distribution equilibrium

$$-K = \frac{dA'_2/dt}{A'_2}. \qquad (4)$$

Then, from *2, 3, 4*

$$\frac{A'_2/A_2}{A'_1/A_1} = \frac{\lambda_{21}}{\lambda_{21} - K} \qquad (5)$$

where A'_2/A_2 and A'_1/A_1 are the specific activities in compartments 2 and 1 respectively.

In normal subjects, λ_{21}, estimated from the rate of disappearance of albumin-I^{131} injected into skin, muscle and subcutaneous tissue, ranged from

0.24–1.2 days^{-1} in one study (4) and in another study (24) averaged about 0.65 days^{-1}, while K is normally about 0.040 days^{-1} (4). Thus the specific activity in the slowly equilibrating extravascular compartment may be overestimated by about 6%. Since the total extravascular albumin is normally about 60% of the total exchangeable albumin, estimates of the latter by the isotope dilution technique are not likely to be in error by more than about 3% in normal subjects.

The error is likely to be somewhat greater in proteinuric subjects but for the specific activity in edema fluid to exceed that in plasma 30-fold (6), K would have to be almost as great as λ_{21}. However, λ_{21} was observed to average about 1.7 days^{-1} (24) in nephrotic subjects and the highest value observed for K in the subjects studied by Gitlin and associates (6) was 0.39 days^{-1}. The highest ratio anticipated between specific activities in edema fluid and plasma should therefore not exceed 1/3 under these conditions.

ADDENDUM

Since the preparation of this brief review there has appeared a paper by Margen and Tarver (25) which merits some comments apropos of considerations already discussed. These authors compared albumin labeled endogenously with S^{35} and albumin labeled *in vitro* with I^{131}. Their conclusions that there is a marked difference in the behavior of these two labeled proteins and that the iodinated protein yields 'no stable half life' would appear to be invalidated by their use of obviously denatured albumin-I^{131} and by considerable evidence to the contrary. The curves and data presented by these authors are similar to those reported previously from this laboratory (4) and by McFarlane (17) for grossly damaged lots of albumin-I^{131} and are quite unlike those for unaltered preparations (4, 13). Since Margen and Tarver estimated total exchangeable albumin from the zero time extrapolation of their plasma curves rather than from the indicator dilution formula, it is also not at all surprising that the values obtained under these circumstances were spuriously high. Values for total exchangeable albumin obtained with satisfactory lots of albumin-I^{131} (4, 13) are, if anything, even lower than those given for albumin-S^{35}. Furthermore, although Margen and Tarver presented values for albumin-I^{131} degradation three times as great as for albumin-S^{35}, their values for the latter (180 mg/kg/day) are only slightly lower than ours with undamaged albumin-I^{131} (4, 13) and these slight differences would appear to be accounted for on the basis of reutilization of the S^{35}.

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INTERPRETATION OF STUDIES OF THE METABOLISM OF ISOTOPICALLY LABELED HORMONES[1]

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ALTHOUGH endocrinologists and biochemists have studied the effects of hormones upon tissue metabolism both *in vivo* and *in vitro* for many years, it has been comparatively recently that techniques have become available to them for the study of the metabolism of the hormones themselves. One of the difficulties has been the fact that the concentration of endocrine secretions in body fluids is so infinitesimal. Recent studies would suggest that the order of magnitude of these concentrations is that of micrograms per cent, ranging from 0.2–0.8 μg% in the case of insulin (1, 2) to from 10–40 μg% in the case of the 17-hydroxycorticoids (3). With plasma concentrations of this order, the analytical problems encountered in the determination of the levels of nonprotein hormones in different physiological and pathological situations are discouraging; for the protein hormones which circulate at such concentrations that even sensitive bioassay procedures are taxed (2), they are well nigh prohibitive.

With the perfection of techniques for the careful radioactive iodination of the protein hormones to specific activities in the order of 5–30 μc/μg of hormone and with the advent of scintillation well counters with sensitivities of the order of 1×10^6 cpm/μc it has been possible to identify these hormones in body fluids and tissues, to estimate their concentrations and to determine their turnover rates. I think it is now well established tht all biological catalysts, i.e. hormones, coenzymes and enzymes, as well as all substrates, participate in the general metabolic turnover which characterizes cellular physiology. Within the past several years we have had some very illuminating studies of hormone metabolism with radioactive iodinated tracers and I shall cite some of them in reviewing the norms which govern the sound interpretation of data forthcoming from such studies.

It is obvious that the usefulness of an isotopically labeled substance in metabolic studies is dependent upon the isotopic substance being *1*) chemically pure, *2*) fully biologically active and *3*) employed in physiological dosage. As has been pointed out by other speakers, iodination of naturally occurring non-iodinated proteins changes the chemical nature of the protein as the tyrosine and histidine moieties take up iodine. The changes with minimal iodination appear to be so slight that they are undetected by study of certain physical-chemical properties such as mobility, sedimentation rate and intrinsic viscosity and by certain biological criteria such as immunologic specificity and specific biological activity. On the other hand, vigorous iodination may alter many of the above criteria and destroy the biological activity (4). The level of iodination which is generally sought in tagging protein hormones is a mean of one atom of iodine per mole of protein. The technique usually employed involves the iodination of the protein hormone with stoichiometric amounts of I_2^{131} at pH's between 7.5 and 9.0 for relatively short periods (ranging from 10 sec. to 30 min., exact duration varying inversely with the pH) followed by dialysis or treatment with an ion exchange resin to remove the excess iodide^{131}. Under these conditions the physical-chemical properties of the protein appear to be unchanged and the biological activity is preserved. It must be remembered, however, that properly conducted bioassays have an error of from 10 to 30% and that the techniques of sedimentation and electrophoresis are subject to errors of the order of 10%. Nevertheless, a combination of assays and analyses of properly iodinated hormone suggests that this material is useful as a tracer for its physiological counterpart.

I should like to begin this discussion of the metabolism of the artificially iodinated protein hormones with a consideration of the metabolism

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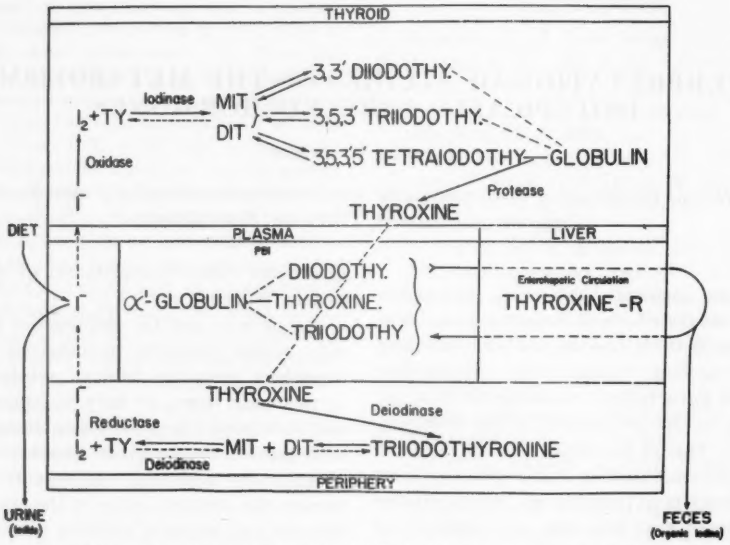

FIG. 1. Metabolism of iodine and physiologically iodinated compounds in the animal body

of the physiologically iodinated non-protein hormone thyroxine. Physiological pathways exist in the mammal for the catabolism of diiodo- and monoiodo-tyrosine which are breakdown products of both thyroxine and artificially iodinated proteins. A summary of thyroid hormone metabolism, as understood today, is presented in figure 1. Inorganic plasma iodide, derived from the diet, is concentrated in the thyroid gland 50- to 150-fold and undergoes oxidation to iodine by an enzyme (5). Since free iodine has never been found in the thyroid gland, it is presumably taken up by tyrosine as soon as formed yielding 3-monoiodo- and 3,5-diiodo-tyrosine. Although Gross and Pitt-Rivers (6) feel that iodination of tyrosine is non-enzymatic, Fawcett and Kirkwood (7) have shown that an enzyme found in thyroid tissue and salivary gland, but not in liver, spleen, heart and skeletal muscle, catalyzes the iodination of tyrosine to monoiodo-tyrosine. These authors postulate further that this enzyme may be involved in the deiodination of monoiodotyrosine in salivary glands and possibly other sites in the periphery where a lower redox potential would favor deiodination.

The iodinated amino acids are then incorporated into thyroglobulin as such, or in the form of thyronine derivatives of which three physiological ones are known: 3,5,3′5′-tetraiodothyronine (thyroxine), 3,5,3′-triiodothyronine (8, 9) and 3,3′-diodothyronine (10, 11). The thyroid gland contains a proteolytic enzyme which, under thyrotropic hormone control, liberates thyroid hormone (mainly thyroxine but also the other thyronines cited above) from thyroglobulin for secretion into the blood. In the plasma, thyroid hormone travels bound to an alpha-1 globulin. In the periphery the hormone is disassociated from plasma protein in order to penetrate cellular membranes and exert its physiological effect. The first dehalogenation product of thyroxine in the periphery, triiodo-thyronine, may indeed be the metabolically active hormone (12). Triiodothyronine is then apparently further degraded to monoiodo- and diiodo-tyrosine and these compounds then deiodinated to yield inorganic iodide and tyrosine. The rate of deiodination of 3,5-diiodo-tyrosine appears to be 3–5 times as rapid as the overall deiodination of thyroxine (13). The iodide appears in the plasma and then may recycle through the thyroid gland or be excreted into the urine by the kidney. In addition to deiodination some of the plasma thyroxine is metabolized via glucuronide formation in the liver followed by biliary excretion into the gut. Much of this thyroxine is reabsorbed but some appears in the stool. The fraction of thyroxine excreted as the glycuronide varies from species to species, being high in the rat, intermediate in the dog and low in man (14).

These pathways are shared, in part, by catabolic products of artificially iodinated proteins.

TABLE 1. FORMULATIONS USED IN ISOTOPIC TURNOVER STUDIES

1. $A = i\left(\frac{\alpha}{\alpha_0} - 1\right)$; 2. $A/C = S$

3. $\ln \frac{\alpha_0}{\alpha_t} = Kt$; 4. $K = \frac{.693}{t^{1/2}} = a/A$

5. $t^t = 1/K = 1.44\, t^{1/2}$; 6. $a = AK$

where:

i = amount of isotopic substance given
α = specific activity of isotopic substance given (CPM/unitweight)
α_0 = specific activity of body pool of isotopic substance at zero time
α_t = specific activity of body pool of isotopic substance at time t
A = exchangeable pool in amount
C = concentration of substance per unit volume
S = apparent space occupied by the substance in the organism
K = fractional turnover rate
$t^{1/2}$ = time for α to decline to $\frac{1}{2}\ \alpha$
t^t = time required to turn over an amount of substance equal to pool size
a = absolute turnover rate in amount per unit time

Although there is no evidence that deiodination of intact proteins occurs in the mammal, there is good evidence to suggest that the iodinated amino acids liberated in proteolysis of these proteins are rapidly deiodinated by the systems mentioned above. The inorganic iodide formed may then be recycled to thyroid hormone in the thyroid gland or excreted in the urine. If quantitative recovery in the urine of iodide liberated in proteolysis is expected, blockage of the thyroid with potassium iodide or thiouracil is required. It also follows from these considerations that if only radioactive label is being followed in tissue studies (total tissue counts, total counts associated with particulates or proteins or counts by radioautography) as evidence of the fate of intact hormone, separate evidence must be adduced to show that the label is truly associated with the hormone at the time of analysis and does not represent the radioactivity of proteolytic products.

If one administers labeled metabolite by vein and follows the specific activity in the plasma as a function of time, a plot of the logarithm of the specific activity of the metabolite against time will reveal one or more linear regression lines as has been noted by the other speakers. From the rate of change of specific activity with time and from the degree of isotopic dilution within the organism, useful information about the metabolic turnover of the substance can be obtained. Standard nomenclature for the constants and parameters of isotope turnover data is desirable. Those suggested by Zilversmit (15) are used for the present discussion and are listed in table 1. The amount of tracer administered (i) in relationship to the pool size (A) should be as small as possible.

In the calculation of pool size, A, the critical value is that of α_0 or the extrapolated zero intercept of the semilogarithmic decay plot. Since this die-away curve is rarely linear and is usually made up of two or three portions of different slope, the problem in arriving at the correct extrapolated value for α_0 is apparent. The initial rapid decline in specific activity generally reflects dilution within the plasma, ± binding to protein, transfer to additional compartments such as the extracellular space and/or intracellular space, as well as metabolism and excretion. This is a period of non-steady state and is meaningless for purposes of extrapolation. If distribution is rapid, the next portion of the decay plot will be linear and will represent the steady state of first order kinetics which characterizes the replacement of isotopic hormone by newly synthesized non-isotopic hormone. From this portion of the curve a meaningful extrapolation to α_0 can be made. If distribution is slower, a third linear portion of lesser slope will finally appear from which the true extrapolation must be made. The final test of adequate mixing is the attainment of parallel rates of change of specific activity in plasma specific activity and total body radioactivity. If disproportionate losses of radioactivity occur in the period of mixing of the body pool of substance with the isotopic tracer due to failure of the administered substance to merge with its physiological counterpart (16), the extrapolated value of the linear portion will give an intercept (α_0) too low and hence an apparent pool size (A) which is too large. Recycling of isotope, on the other hand, will decrease the slope of the die-away curve and give values for biological decay (t^t and $t^{1/2}$) which are too slow.

The physiological space (S) is obtained by dividing the pool size (A) by the plasma concentration (C). This calculation assumes that isotope dilution is complete, that plasma concentration and pool size have been constant throughout the experiment, and that the substance is distributed throughout the space at plasma concentration.

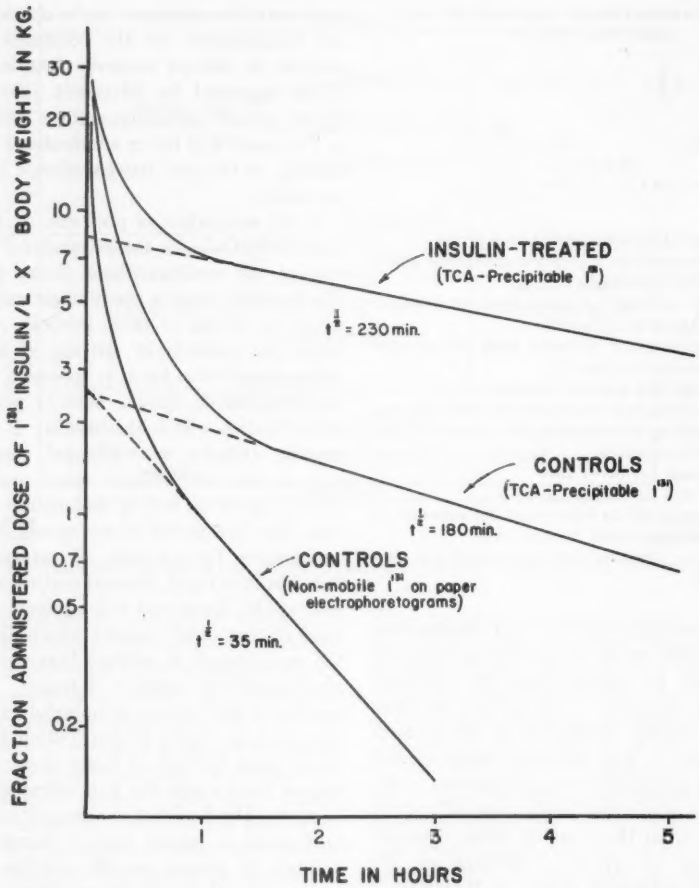

FIG. 2. Biological decay of insulin-I^{131} in patients with and without prior insulin treatment. After Berson *et al.* (1).

Only in a limited number of situations are all three provisos true, and only in these situations does S have a physiological significance.

The fractional turnover rate (K) is the fraction of the pool renewed per unit time and may be calculated from the linear portion of the decay curve in the steady state. Its reciprocal is the turnover time (t_t), or the time required to turn over an amount of metabolite equal to the pool size. The absolute turnover rate, a, is determined by multiplying the fractional turnover rate times the pool size and its accuracy therefore will depend upon having accurate values for both K and A.

The necessity for employing suitably prepared and purified labeled hormone for the determination of the rate of metabolic turnover in the animal body cannot be overemphasized. McFarlane and his colleagues (16) have shown that improperly iodinated albumin is preferentially degraded during the period of equilibration prior to establishment of the slow exponential decay curve and if this latter portion is extrapolated to zero time the pool size obtained may range up to four times that obtained with properly iodinated albumin. We have noted a similar artefact in the study of the metabolism of *l*-thyroxine-I^{131} in euthyroid and thyroid-fed hyperthyroid dogs employed in a study of the effect of excess thyroid hormone upon cardiac metabolism (17). Hyperthyroid dogs given *l*-thyroxine-I^{131} by vein gave extrapolated value of the exponential portion of

the die-away curves which were indicatory of a space of distribution twice as large as that obtained in normal dogs accompanied by decreased total urinary iodide excretion. Since this phenomenon is not seen in hyperthyroid man given endogenously synthesized thyroid hormone (18), the explanation may reside in the fact that *l*-thyroxine (apart from circulating *l*-thyroxine-protein complex) may be so rapidly metabolized to its glucuronide and excreted via the bile in the hyperthyroid animal, that a sizeable percentage of the administered radioactive thyroxine is excreted before it becomes fully assimilated into the serum protein-bound iodine pool. Albert and Keating (19) noted extremely rapid accumulation of radioactivity in the liver (30% of the dose in 1 min.) following administration of *l*-thyroxine-I^{131} to rats with subsequent secretion into the gastrointestinal tract. Such an event would increase the apparent space of distribution by a large factor and may account for the enlarged thyroxine space of hyperthyroid dogs. It also should be pointed out that an active enterohepatic circulation of thyroxine would also increase the biological half-life in the same way as does any other process of recycling of isotope. All of these factors must be considered in the interpretation of studies of iodinated-hormone turnover.

As far as I have been able to determine, four protein hormones have been iodinated for metabolic studies. These are insulin (20), lactogenic hormone (21), adrenocorticotrophic hormone (20, 22) and glucagon (23). Some very interesting results have been obtained in the study of the metabolism of insulin by Berson and his colleagues (1). Figure 2 presents the results of administration of insulin-I^{131} to humans with and without prior insulin treatment. A function of the specific activity of plasma insulin is plotted against time. The specific parameter used (fraction administered dose I^{131}-insulin per liter × body weight in kilograms) is the reciprocal of the space of distribution in terms of percentage of body weight. The curves presented are the average curves for from 12 to 20 persons in each group. The top curve represents the rate of change of trichloracetic acid precipitable iodine^{131} in the plasma of diabetics and other non-diabetic patients who had received insulin prior to the experiment. The apparent half-life of insulin in these patients was 230 minutes and the space of distribution about 12% of the body weight. In control patients who had not received insulin prior to the experiment, an appreciably larger space of distribution (45% of body weight) and a shorter half-life (180 min.) were observed. When paper electrophoretograms were made of the plasma from each group of subjects at intervals after insulin-I^{131} administration, it was observed that *1*) unbound insulin appeared to be adsorbed to the paper at the origin and did not migrate; *2*) large amounts of radioactivity migrated with gamma globulin in the insulin-treated group and *3*) smaller amounts of radioactivity migrated non-specifically with all of the plasma proteins in both groups. It was then

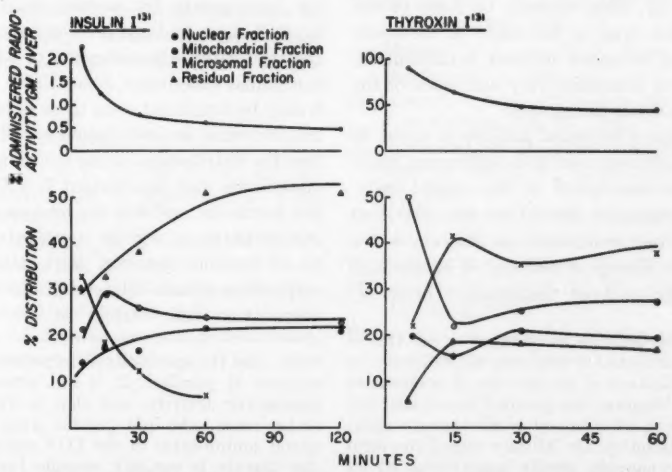

FIG. 3. Distribution of radioactivity in liver intracellular components after administration of insulin-I^{131} as compared with thyroxine-I^{131}. After Lee and Williams (29).

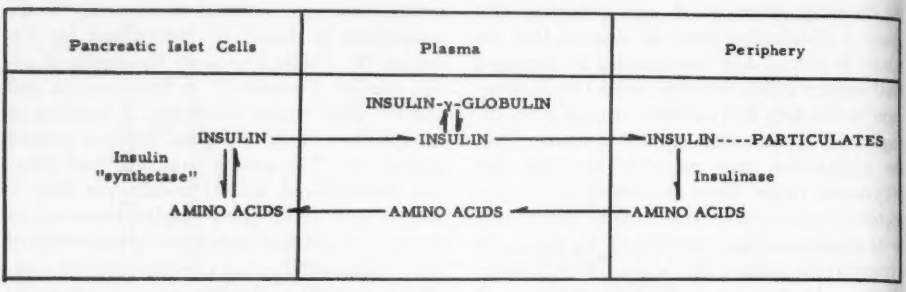

FIG. 4. Metabolism of insulin in the animal body.

established that tagged insulin combined reversibly with gamma globulin in patients who had previously received bovine insulin and this accounted for the prolonged retention and small space of distribution noted in this group. It was further noted that the variable amounts of radioactivity migrating with all of the plasma proteins represented 'changed insulin,' possibly through self-radiation, and constituted an artefact. When the non-mobile I^{131} on the electrophoretograms was plotted against time for the normal group, a third and lowest curve with a half-life of 35 minutes was obtained. This, in all probability, represents the true turnover rate of insulin in the normal subject. By assuming an insulin requirement and hence daily turnover rate in the depancreatized human of 48 units/day and applying the equations previously outlined, it was calculated that the plasma concentration was 0.25 μg%, which is of the same order as determined by bioassay (2). This example of some of the problems which arise in the study of metabolic turnover of an iodinated hormone is particularly good because it illustrates very well some of the pitfalls which await the unwary.

If insulin has a biological half-life of about 35 minutes it is obvious that it is undergoing rapid synthesis and destruction in the animal body. Mirsky demonstrated some time ago (24) that extracts of liver contained an enzyme which destroyed the biological activity of insulin and designated the enzyme 'insulinase.'[2] More recently he and coworkers (25) have found that iodinated amino acids are liberated from insulin-I^{131} by this enzyme and that the kinetics of the proteolysis followed the Michaelis-Menten formulation. Precise specificity for insulin was not demonstrated, however, with ribonuclease, pepsin and prolactin undergoing some splitting. Tomizawa *et al.* (26) have adduced additional evidence that the insulin inactivating enzyme also splits a-corticotropin, a-casein, growth hormone and glucagon.

Stadie and his colleagues discovered that insulin is bound to tissues (27) and have concluded that this binding is essential for its biological activity (28). Another aspect of insulin metabolism, therefore, is the extent of binding to cells in the periphery. Lee and Williams (29) injected iodinated insulin into whole animals and determined the amount of radioactivity in liver homogenate and in the particulates isolated from the homogenate by sucrose fractionation. In figure 3 these findings are compared with those observed after administration of *l*-thyroxine-I^{131} in a similar experiment. Since NaI^{131} is not bound, it may be concluded from these data that insulin and thyroxine are both bound to cell particulates, that the distribution among nuclei, mitochondria, microsomes and supernatant is different for the two hormones, and that the time course of change in distribution of activity represents either transfer of hormone between particulates or the incorporation of metabolic end-products of hormone degradation into certain of the particulates.

[2] I think the decision on whether or not this is an appropriate term for this enzyme will have to wait for purification of the enzyme. I understand that Martha Vaughan has purified insulinase, but I do not know to what extent or whether she actually has crystalled it. Dr. Mirsky coined the term 'insulinase' to connote insulin inactivation by an enzyme. The evidence to date is based upon incubation of insulin with partially purified liver extract, and the specificity is hypothetical until the enzyme is purified. It is not proved that this proteolytic activity, and that is what it appears to be, since iodinated insulin gives rise to iodinated amino acids in the TCA soluble portion of the digests, is entirely specific for insulin. The fact that it is not entirely nonspecific, I think, is established.

Further studies to clarify the peripheral metabolism of both hormones with additional controls (3,5-diiodotyrosine-I^{131}) are needed.

A summary of insulin metabolism, as revealed with isotopic tracers, is shown in figure 4. Insulin, synthesized in the pancreatic islet cells from amino acids, is secreted into the plasma where it travels to the periphery and is absorbed or adsorbed to cellular membrane and particulates and exerts its physiological function (28, 30). Enzymes in the periphery degrade insulin to amino acid end products which reenter the amino acid pool and may serve again as insulin precursors in the pancreas. If commercial insulin is administered to man, an 'antibody-like' response is observed with resultant binding of the administered insulin to gamma globulin which dissociates over a prolonged period of time. Whether this response represents a species specificity for insulin or reflects the appearance of subtle changes in structure of bovine or porcine insulin during isolation has not yet been determined.

In conclusion I should like to say that this presentation was intended to review the metabolism of iodinated proteins and amino acids in the body, to point out the interdependence of the parameters of biological turnover determined from decay curves after administration of purified hormone, and to illustrate, with studies of one hormone, insulin, some of the variables which complicate the interpretation of turnover data. Finally, I should like to say that although there are numerous precautions which must be taken in such experimentation, investigators should be encouraged to explore further the metabolism of the protein hormones with the aid of this technique.

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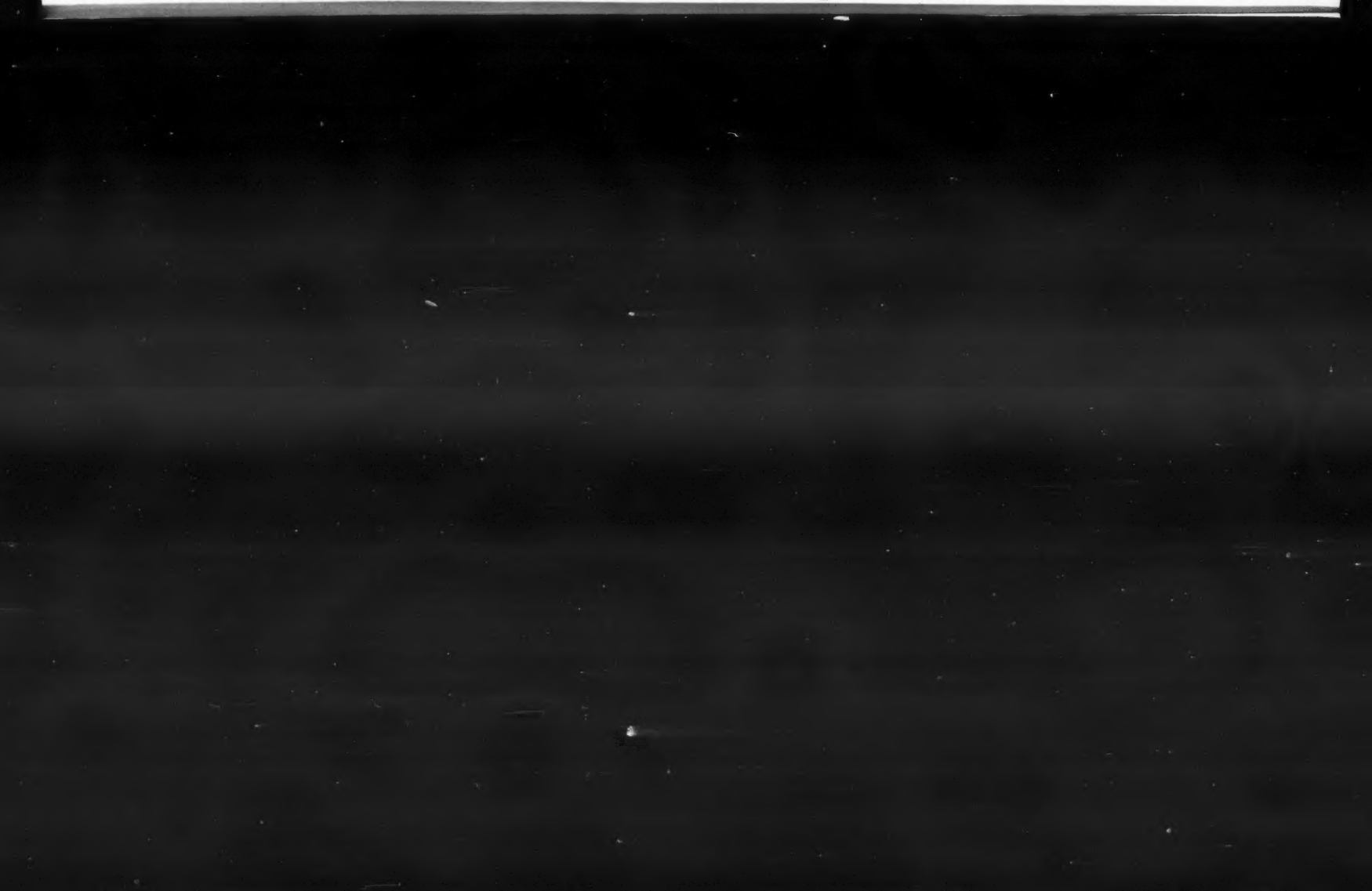

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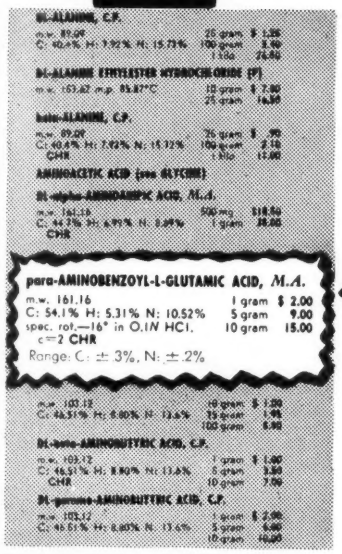

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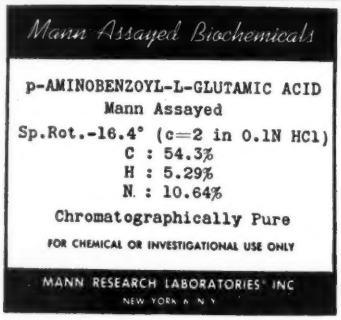

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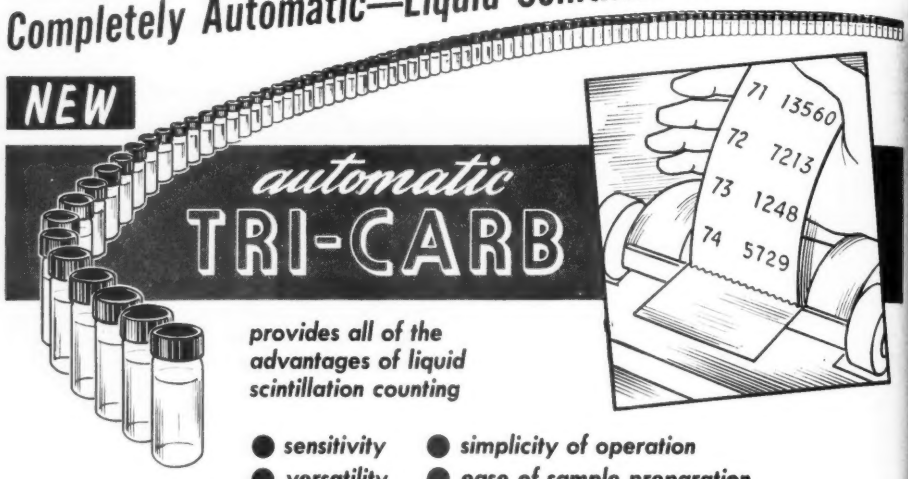

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